SENESCENCE OF CYTOTOXIC LYMPHOCYTES: FUNCTIONAL CHANGES AND ROLE OF CMV

UNIVERSITY OF CORDOBA

DEPARTMENT OF CELL BIOLOGY, PHYSIOLOGY AND IMMUNOLOGY

INSTITUTO MAIMÓNIDES DE INVESTIGACIÓN BIOMÉDICA DE CÓRDOBA (IMIBIC)

Fakhri M. F. Hassouneh

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Doctoral Thesis presented by

Fakhri M. F. Hassouneh,

supervised by

Dr. **Rafael Solana Lara**

Dra. **Alejandra Pera Rojas**

Córdoba, 2017

TÍTULO DE LA TESIS:

SENESCENCIA DE LINFOCITOS CITOTÓXICOS: CAMBIOS FUNCIONALES Y PAPEL DEL CMV.

DOCTORANDO/A: Fakhri M. F. Hassouneh

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

D. Fakhri M. F. Hassouneh inició la realización de su Tesis Doctoral en 2013 en el Programa de Doctorado de Biomedicina. La fase experimental ha sido realizada en el IMIBIC y durante este tiempo ha adquirido un amplio conocimiento científico y tecnológico en inmunología, en particular en el estudio de las células T citotóxicas en el contexto del envejecimiento y la infección por CMV. Así mismo, el doctorando ha aprendido a diseñar y desarrollar un proyecto de investigación original, realizar un análisis crítico de nuevas ideas y comunicarse con la comunidad científica en nuestro ámbito de conocimiento. La tesis presentada por el doctorando es una tesis por compendio de artículos y mención internacional donde se ha realizado una estancia de cuatro meses en el laboratorio del Dr. Pawelec, director del grupo de investigación: Tübingen Ageing and Tumour Immunology group (TATI), en Eberhard Karls Universität, Tübingen, Alemania. Los resultados obtenidos de este trabajo han sido publicados en revistas científicas de reconocido prestigio internacional en el campo del la investigación en inmunología y enfermedades infecciosas: *Frontiers in Immunology, Mechanisms of Ageing and Development* y *International Journal of Molecular Sciences* y también han sido presentados en diferentes congresos de ámbito nacional e internacional.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 30 de Octubre de 2017

Firma del/de los director/es

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SUPORTING INFORMATION

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GLOSSARY

(AIDS) Acquired Immune Deficiency Syndrome

(ALL) Acute Lymphoblastic Leukemia

(AR) Allergic Rhinitis

(CCA) Chronic Chorioamnionitis

(CD1) Cluster of Differentiation 1

(CMV) Cytomegalovirus

(CTLA-4) Cytotoxic T lymphocyte associated antigen 4

(CTLs) Cytolytic T lymphocytes

(CXCR3) CXC chemokine Receptor 3

(DAP12) DNAX acti-vating protein of 12 kDa

(DN) Double Negative

(DP) Double Positive

(EM) Effector Memory

(Eomes) Eomesodermin

(glycoepitope) glycan carbohydrate epitope

(HCV) Hepatitis C Virus

(HIV) Human Immunodeficiency Virus

(IFN-γ) Interferon gamma

(IL) Interleukin

(IL12Rβ1) Interleukin 12 receptor β1

(iNKT) Invariant NKT

(IRP) Immune Risk Phenotype

(KIR) killer immunoglobulin-like receptors

(LLT1) Lectin-Like Transcript 1

(MCMV) Murine Cytomegalovirus

(MHC) Major Histocompatibility Complex

(NK) Natural killer

(NKRs) Natural killer cell receptors

(NKT) Natural killer T

(PD-1) Programmed death-ligand 1

(PE) Phosphatidylethanolamine

(PS) Phosphatidylserine

(SIV) Simian Immunodeficiency Viruse

(T_{CM}) T lymphocyte Central Memory

(TCR) T Cell Receptor

(T_{EM}) T lymphocyte Effector Memory

(T_{EMRA}) T lymphocyte Effector memory RA

(TNF-α) Tumor necrosis factor-alpha

(αβ) Alpha beta

(γδ) Gamma delta

INDEX

1- RESUMEN/ SUMMARY……9

2- GLOBAL INTRODUCTION……..13

3- HYPOTHESIS and OBJECTIVES……22

4- SUBJECTS, MATERIALS AND METHODS, RESULTS AND DISCUSSION……23

4.1. Effect of age and latent CMV infection on CD8+CD56+ T cells (NKT-like) frequency and functionality. **Fakhri Hassouneh**, Carmen Campos, Nelson López-Sejas, Corona Alonso, Raquel Tarazona, Rafael Solana, Alejandra Pera. https://doi.org/10.1016/j.mad.2015.12.003.

4.2. Differential Effect of Cytomegalovirus Infection with Age on the Expression of CD57, CD300a, and CD161 on T-Cell Subpopulations. **Fakhri Hassouneh**, Nelson Lopez-Sejas , Carmen Campos , Beatriz Sanchez-Correa, Raquel Tarazona , Rafael Solana, Alejandra Pera. Front Immunol 8, 649.2017 Jun 02.

4.3. Effect of Cytomegalovirus (CMV) and Ageing on T-Bet and Eomes Expression on T-Cell Subsets. **Fakhri Hassouneh**, Nelson Lopez-Sejas , Carmen Campos , Beatriz Sanchez-Correa , Raquel Tarazona , Alejandra Pera and Rafael Solana. Int. J. Mol. Sci. 2017, 18(7), 1391; doi:10.3390/ijms18071391.

5- GENERAL DISCUSSION……74

6- CONCLUSIONS……76

7- FUTURE PERSPECTIVES……..78

8- REFERENCES……..79

9- OTHER PUBLICATIONS……87

1. RESUMEN

Inmunosenescencia es el deterioro de la inmunidad como resultado de los cambios asociados a la edad. Los cambios relacionados con la edad se caracterizan por cambios fenotípicos y funcionales de las células inmunes que se han asociado clínicamente con la disminución de la eficacia de las vacunas, aumento de la frecuencia y gravedad de las enfermedades infecciosas, así como un aumento de la incidencia de cáncer, trastornos inflamatorios crónicos y autoinmunidad. La inmunosenescencia afecta tanto a la inmunidad innata como a la adaptativa, siendo esta última la más afectada en este proceso. Existe evidencia de la asociación de la infección crónica por citomegalovirus humano (CMV) con un envejecimiento acelerado del sistema inmunológico y varias enfermedades relacionadas con la edad, por lo que se le ha considerado tradicionalmente un factor importante implicado en el proceso de immunosenescencia. En este trabajo de tesis me he centrado en estudiar el efecto de la edad y la infección por CMV sobre la frecuencia, función y fenotipo de diferentes subconjuntos de células T. Nuestros resultados mostraron que tanto la frecuencia como la respuesta a Staphylococcal Enterotoxin B (SEB) de la subpoblación de linfocitos T CD8+CD56+ (NKT-like), así como la polifuncionalidad de dichas células, aumenta con la edad en el contexto de la infección por CMV. Además, las células NKT-like que expresan CD57 se expanden en individuos CMV-seropositivos y son más polifuncionales que las CD57−. Por otro lado, la infección por CMV tiene un efecto diferencial con la edad sobre la expresión de los marcadores CD57, CD300a y CD161 en diversos tipos de células T. Pudimos observar que en todas las subpoblaciones de células T estudiadas, CD57 y CD300a aumentan con la edad en individuos CMV-seropositivos, mientras que CD161 disminuye. También se observamos que, en individuos jóvenes CMV-seropositivos, la expresión de CD57 aumenta sólo en las células T CD4+ y CD8+, y la de CD300a en CD4+ y CD4−CD8− (DN). Además, la expansión de células T CD57+ que coexpresan CD300a es un sello de la infección por CMV. Así mismo, nuestros resultados mostraron que el porcentaje de células T que expresan Eomes y T-bet es mayor en las células T CD8+ y $CD4^{hi}CD8^{lo}$ que en las células

T CD4+. Los linfocitos CD57+ siempre expresan T-bet con o sin Eomes, pero nunca al contrario. También observamos que las células T CD4+CD57+ sólo se encuentran en individuos CMV-seropositivos independientemente de su edad. La infección por CMV también se asoció a un aumento del porcentaje de células T $CD4^{hi}CD8^{lo}$ T-bet+. En individuos CMV-seropositivos, la edad se asoció a un aumento en los niveles de T-bet y Eomes en la subpoblacion CD8+. Estos resultados demuestran que la edad y la infección por CMV tienen un profundo impacto en el fenotipo y función de las células T y que el efecto del CMV varía según el tipo celular y la edad del individuo. Por tanto, el presente trabajo subraya la importancia de tener en cuenta ambos factores (edad y CMV) en cualquier estudio del sistema inmune y su deterioro.

SUMMARY

The impairment of the immune response as a result of age-associated changes has been called immunosenescence. Age related changes are characterized by phenotypical and functional changes of the immune cells that have been associated clinically with decreased efficacy of vaccines, an increase in the frequency and severity of infectious diseases and an increased incidence of cancer, chronic inflammatory disorders and autoimmunity. Both innate and adaptive immunity are involved in immunosenescence, where the adaptive response is affected the most in this process. There is an extensive data showing that latent persistent human cytomegalovirus (CMV) infection is associated with accelerated ageing of the immune system and with several age-related diseases. In this thesis work I have focused on studying the effect of age and infection by CMV on the frequency, function and phenotype of different T-cell subsets. Our results showed that the percentage, response to Staphylococcal Enterotoxin B (SEB) and the polyfunctionality of CD8+CD56+ T cells (NKT-like cells) increase with the combination of both CMV and age. NKT-like CD57+ cells expand with CMV and are more polyfunctional than their CD57− counterpart. Furthermore, CMV latent infection has a differential effect with age on the expression of CD57, CD300a, and CD161 markers on T-cell subsets. We observed that in all T-cell subsets studied, CD57 and CD300a increase with age in CMV-seropositive individuals; while CD161 decreases. We also found that in CMV-seropositive young individuals, CD57 is increased only in CD4+ and CD8+ T cell subsets and CD300a in CD4+ and CD4−CD8− (DN) cells. Also, the expansion of CD57+ T-cells co-expressing CD300a is a hallmark of CMV infection and is further increased by age. In addition, our results showed that the percentage of T-cells expressing Eomes and T-bet was higher in CD8+ T-cells and in $CD4^{hi}CD8^{lo}$ than in CD4+ T-cells. Moreover, CD57+ T-cells were T-bet+ with or without Eomes but never Eomes+T-bet−. We also found that CD4+CD57+ T-cells always coexpress T-bet and are only found in CMV-seropositive individuals independently of their age. The percentage of $CD4^{hi}CD8^{lo}$ T-cells expressing T-bet was associated with CMV-seropositivity. Moreover, Age is associated with the increase of T-bet and Eomes in CD8+ T-cells. These results, demonstrate that age and CMV infection have a

profound impact on the immune phenotype and function of T-cells and that the effect of CMV also can vary depending on the cell type and the age of the individual. Thus, our results highlight the importance of taking into account both age and CMV serostatus in any study regarding the analysis of the immune system and its deterioration.

2. GLOBAL INTRODUCTION

The immune system of an organism is a fundamental element of the defense mechanism, directed at fighting pathogenic stress through its two “lines of defense”. The innate immunity represents the first line of defense and is non-specific; whereas the adaptive immunity involves a more complex mechanism as is antigen-specific. Thus, once a specific antigen to a given pathogen is recognized, the adaptive immune system responds with a high degree of specificity and memory, enabling the host to produce a more rapid and efficient immune response. The adaptive response is based principally on the expression of the antigen-specific receptors on the surfaces of T- and B-lymphocytes (1-3)

Naïve T-cells develop in the thymus, which is a primary lymphoid organ of the immune system (4). After leaving the thymus, naïve T-cells go to the periphery where they will remain naïve or differentiate to memory phenotype depending on antigen encounter (5). T lymphocytes express a T-cell receptor (TCR) that is composed of either alpha beta (αβ) or, less frequent, gamma delta (γδ) chains. TCRαβ+ T-cells subsequently develop to be either CD8+ or CD4+ that are restricted by Major Histocompatibility Complex (MHC) class I or II molecules respectively. These subsets display different functions and further subdivision (6). CD4+ T-cells generally provide help to other immune cells through cytokine secretion and expression of specific surface molecules. While, CD8+ T-cells are cytotoxic and kill directly infected cells and tumor cells (7). It has been defined several differentiation stages of T-cells according to the expression of the surface markers CD45RA and CCR7: Naïve T lymphocytes (CD45RA+ CCR7+), central memory T lymphocytes (T_{CM}: CD45RA−CCR7+), and two memory-effectors T lymphocyte subsets (T_{EM}: CD45RA−CCR7− and T_{EMRA}: CD45RA+ CCR7−) (8). The differentiation pathways towards the effector and memory T-cell phenotypes are tightly regulated by the transcription factors T-bet and Eomesodermin (Eomes) (9-11). Indeed, in human CD8+ memory T-cells, has been shown that higher expression levels of T-bet are associated with effector and some T_{EM} cells; whereas lower levels of T-bet correlate with T_{CM} and some T_{EM} cells. On the other hand, Eomes

expression was found in the majority of effector and T_{EM} cells and was found to be higher in T_{EM} subsets (12).

A special subset of lymphocytes that exhibit features of both T-cells and natural killer (NK) cells are the so called natural killer T (NKT) cells that express NK markers such as CD56. These cells are divided into two major subtypes: cluster of differentiation 1 (CD1d)-dependent cells, which have a semi-invariant TCR, frequently called NKT-cells or invariant NKT (iNKT) cells and CD1d-independent NKT-cells expressing CD8+ and CD56+ referred as NKT-like cells (13). It has been shown that CD8+CD56+ NKT-like cells display high tumor cytotoxic capacity, cytokine production and EM or effector phenotype (14, 15). A minor periphery T-cell subset that express neither the CD4 nor CD8 surface molecules are classed as CD4−CD8− double-negative (DN) T-cells (16). These cells are different from the DN T-cells found in the thymus in early stages of maturation (17). The majority of DN T-cells express TCR-γδ and account for approximately 1-10% of human peripheral T-cells (16, 18). An important role of DN T-cells was reported in various diseases such as graft-vs-host disease (19), autoimmune diseases (20), parasite infection (21), and Simian Immunodeficiency Viruse (SIV)/ Human Immunodeficiency Virus (HIV) infections (22, 23). Moreover, it was reported that these cells recognize non-MHC ligands (18) and predominantly display an effector memory phenotype. Furthermore, under stimulation these cells are polyfunctional (24). Polyfunctionality is the cell ability of producing simultaneously two or more immune mediators such as: Interferon gamma (IFN-γ), Tumor necrosis factor-alpha (TNF-α) and CD107 (a marker of cell degranulation) (25, 26). Polyfunctionality of immune cells has been traditionally considered a sign of a good immune response (27).

More recently it has been described another rare T-cell subset. The extra-thymic double positive (DP) CD4+CD8+ T-cells that exist in healthy individuals of different ages and their percentage has been found to be increased in inflammatory autoimmune diseases (28). Peripheral DP T-cells have a mature phenotype and do not express thymic markers and it has been shown that these cells are antigen specific for viral infections (28, 29). Both co-receptors (CD4 and CD8) are functional in peripheral DP T-cells, conferring these cells new functions. Several subsets of DP peripheral T-cells have been described based on the level of expression of CD4 and CD8 molecules and

on the CD8 chain composition (αα, αβ), indicating a differential origin of these subsets. Of the different DP T-cell subsets described, $CD4^{hi}CD8^{lo}$ (αα) cells have been shown to be terminally differentiated effector CD4+ T-cells (28).

During ageing, the immune system undergoes alterations and changes that can affect the immune response, a term known as immunosenescence. These age-related alterations of the immune system are observed in human and many other species and have been shown to contribute to decreased vaccine responses, increased susceptibility and severity to infectious disease, autoimmunity and cancer. Among those changes, the alterations in number, phenotype and functional capacity of immune cells have been associated with a greater mortality of aged population (30, 31).

A variety of immune cells, including both innate and adaptive, are affected by age. However not all cells are affected in the same way, with the same speed or in the same direction. The most notable changes are observed in the adaptive immune response, and more specifically, within the T-cell compartment (32, 33). T-cells are considered the base of the adaptive immunity and have an important impact on the overall immune response as they are the effectors and regulators of the immune response (34). Several studies have shown that decreased numbers of naïve T-cells and increased numbers of antigen-experienced memory T-cells contribute to the decline of the adaptive immunity (35). Naïve T-cells decrease is mainly a result of the involution of the thymus with age, which is believed to associate with poor immunity due to its impaired ability to activate and differentiate with age (36). This decreased thymic output is compensated by an increase in the homeostatic proliferation that helps to maintain the T-cell compartments (37). Thus, it has been suggested that homeostatic dysregulation contributes to the age-related defects in the immune system, due to the distortion of the naïve repertoire in the elderly (37).

In the elderly there is a redistribution of the CD8+ T-cell subpopulations. This phenomenon is characterized by the expansion of lymphocytes with T_{EM} phenotype with low CD27 and CD28 expression, high expression of NK receptors, decreased oligoclonal expansions repertoire and additionally, accumulation of virus specific CD8+

T lymphocytes (38, 39). CD28 co-stimulatory molecule is crucial for a complete T-cell activation, proliferation and survival. Its loss is associated with increased susceptibility to infections and a poor immune response to vaccination in older people (40-43). Nevertheless, once a naïve T-cell differentiates to activated memory cells, it is less reliable on CD28 mediated co-stimulation (44).

It has been demonstrated that infection with human cytomegalovirus (CMV), which is a persistent herpes virus, is associated with age-related immune dysfunction of the T-cells. CMV has a great impact on the immune system causing alterations in the numbers and proportions of peripheral immune cells of infected individuals. For that reason it has traditionally been considered as an important factor in immunosenescence (45, 46). In fact, studies regarding immunosenescence have revealed the existence of a set of immunological markers that are associated with increased morbidity and lower survival in elderly individuals, which have been called immunological risk phenotype (immune risk phenotype, IRP). This IRP was defined in a cohort of Swedish elderly, and includes low CD4:CD8 ratio (normal is >1; *IRP* is <1) due to an increase in CD8+CD28− T-cells, clonal expansions of CD8+ T-cells and CMV seropositivity and has been associated with increased risk of death (38, 47).

CMV is a prevalent human pathogen, infecting 60 - 70% of the population in industrialized countries and up to 100% in emerging countries (33). CMV primary infection and its long-term persistence are normally asymptomatic in immunocompetent individuals. However, it can be life threatening in immunocompromised individuals (such as HIV/ Acquired Immune Deficiency Syndrome (AIDS) patients and transplant patients) (48).

Following primary infection, CMV induce lytic and latent infections to establish lifelong persistence in human hosts (49-51). CMV undergoes three stages in lytic infection which are divided into immediate early, early, and late phases. After primary infection, CMV usually establishes viral latency in CD34+ hematopoietic progenitors cells of the bone marrow and myeloid lineage cells (52, 53). However, CMV can respond to specific stimuli and be able to reactivate in latent phase (53-55). One of the strategies that CMV has developed to survive and persist in infected individuals is

blocking MHC class I and II antigen presentation, which allows the virus to escape host antiviral responses (56). The adaptive immune system pays a high price to maintain CMV in a latent phase and prevent productive (lytic) infection. The need of a sustained long-lasting adaptive immunity in healthy individuals is crucial and specially in immunocompromised individuals where CMV can lead to serious clinical conditions (57-61).

It has been shown that CMV-specific memory T-cells tend to gradually increase in number with age. In elderly CMV infected individuals (CMV+) up to 50% of CD8+ and 30% of CD4+ T-cells can be CMV-specific (62-64). CMV+ individuals have an increased number of CD28− within CD4+ and CD8+ T-cell compartments along with fewer naïve T-cells. This phenomenon seems to be more dramatic in the CD8+ compartment, resulting in a net increase in CD8+ T-cells (41, 65-67). Moreover, old CMV+ individuals have higher levels of T_{EM} and T_{EMRA} cells in the CD4+ and the CD8+ T-cell compartments compared to CMV− individuals (68). Other studies have also reported telomere length shortening in the CD8+ T-cell subset due to the accumulation of T_{EMRA} CD4+ and CD8+ T-cells associated with CMV infection. However, short telomere length has been also correlated with age, suggesting that CMV associates with the acceleration process of immunosenescence of the immune system (69).

Taken together these data suggest a deleterious impact of CMV on host immunity and its role in enhancing the aging process. However, controversial results have suggested the possibility that CMV might be beneficial to the host immune system. A study in a murine model reported that murine CMV (MCMV) provided a significant protection from an influenza virus in young infected mice compared with uninfected animals (70). Also, MCMV infection provided resistance to bacterial pathogens infection in MCMV+ mice with increased levels of IFN-γ (due to MCMV infection) compared to MCMV− mice (71). Furthermore, in young and middle-aged individuals, CMV associates with the expansion of highly polyfunctional CD57+ T-cells (both CD4+ and CD8+) in response to stimulation (25, 26, 46, 72).

CD57 marker also called HNK-1, LEU-7, orL2, is a terminally sulfated glycan carbohydrate epitope (glycoepitope) that was firstly identified on Human NK cells in 1981

(73, 74). Although first characterized as an NK cell marker, CD57 expression on T-cells is considered as a marker of terminal differentiation (25, 26, 75). It has been suggested that CD8+CD57+ T-cell subpopulation has an important role on age-related changes in the immune system status and in various diseases or conditions, associated with chronic immune activation such as cancer, autoimmune diseases, chronic intracellular infections and other diseases (76, 77). A progressive oligoclonal accumulation of CD8+CD28−CD57+ T-cell population was shown to be associated with ageing (78-80) and was described that a decrease of CD28 is associated with an increase of CD57 expression (78, 81). Under chronic antigen stimulation, CD8+CD57+ T-cells express NK cell receptors (NKRs) such as killer immunoglobulin-like receptors (KIR), and CD56 (82), as well as cytotoxic molecules such as perforin, granzymes, granulysin and have high cytotoxic potential (83-88).

Moreover, it has been reported that CD8+CD57+ T-cells accumulate frequently in individuals with various forms of cancer (76). Also, clear examples of CD8+CD57+ T-cells expansion was seen in many chronic viral infections such as HCMV (89), HIV (83), and hepatitis C virus (HCV) (90), probably as a result of persistent antigenic stimulation. Indeed, although CD57+ T-cells have been traditionally considered as a marker of ageing, more recently it has been suggested that CMV is a main driver of their expansion and that they are a hallmark of CMV infection (25, 26). Furthermore, it has been shown that expression of several functionally active NKRs on the surface of T-cell subsets might regulate the functional activity of these cells (82, 91). Indeed, there is evidence that CD3+CD56+ T-cells produce higher levels of pro-inflammatory cytokines (IFN-γ a TNF-α) and CD107a in CMV+ compared with CMV− subjects (92). Also, it was reported that the vast majority of T-cells expressing NKRs are included particularly within the CD8+CD28−CD57+ T-cell population (14, 87, 93). In light of these results and as there are very few studies regarding the effect of age and CMV infection on NKT-like responses to pathogens, we decided to further characterize these cells in the context of ageing and CMV infection. Thus, the first aim of the present thesis was to study the NKT-like cells response to Staphylococcal Enterotoxin B (SEB), in relation to the polyfunctionality marker CD57. This objective was addressed in the article **“Effect of age and latent CMV infection on CD8+CD56+ T cells (NKT-like) frequency**

and functionality". Mech Ageing Dev.2016 Sep;158:38-45.doi:10.1016/j. mad. 2015. 12.003. Epub 2016 Jan 2.

The expression of activating and inhibitory receptors such as DNAX activating protein of 12 kDa (DAP12), cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and programmed death-ligand 1 (PD-1) by immune cells has an important role in regulating the immune system, resulting in a competent immune response (94-97). CD300a is an inhibitory receptor that belongs to the 7 members human CD300 family that are located on chromosome 17 (98) and has been shown to be expressed in T and B cells (99, 100) as well as in all NK cells (101). The CD300 family members regulates a diverse array of immune cell processes and are involved in the pathogenesis of several diseases such as allergies, leukemia (98, 102, 103) and have been proposed as possible biomarkers, as well as potential targets for therapeutic purposes (102). CD300a binding to phosphatidylethanolamine (PE) and phosphatidylserine (PS) is important for the regulation of the removal of dead cells (104). Moreover, CD300a was shown to be differentially expressed in acute lymphoblastic leukemia (ALL) (105), while interaction with PS was shown to inhibit tumor cell killing by NK cells (106). CD300a seems to contribute to the B-cell dysfunction observed in HIV-infected patients as its expression on B cells is deregulated during HIV infection (107). Furthermore, activated CD4+ T-cells expressing CD300a produce higher levels of T helper (Th1)-associated cytokines and up-regulate the transcription factor Eomes. However, both CD300a+ and CD300a– cells up-regulated T-bet to similar levels (108). An expansion of CD8+CD300a+ T lymphocytes was found in pregnant women with chronic chorioamnionitis (CCA) and the expression of CD8+CD300a+ has been shown to be associated with better cytotoxic function (100). Another inhibitory receptor, CD161 is a homodimeric C-type lectin type II transmembrane glycoprotein expressed by the majority of NK cells (109) and in a subset of T-cells (110). It has been described that CD161 have both inhibitory (111-113) and costimulatory (111, 113, 114) effects through its binding to its ligand lectin-like transcript 1 (LLT1). In human, CD161 expression was proposed to be a hallmark of Th17 cells, which originally originate from CD4+CD161+ naive T-cell progenitors (115). However, a study by Maggi et al, showed that the expression of CD161 is not a hallmark exclusive to CD4+ Th17 cells, but it could be generically applied to all human

Interleukin (IL)-17-producing T-cells, including CD4+, CD8+, and CD4−CD8− cells (116). Furthermore, CD161+ T-cells have an enhanced innate ability to respond, in a TCR-independent manner, to cytokine stimulation (IL-12 plus IL-18) (6). Also, it was found that in peripheral blood of patients suffering from rheumatic diseases, CD161+CD8+ T-cells were decreased (117). However, the frequency of CD3+CD161+, CD4+CD161+ and CD8+CD161+ T-cells were found significantly higher in allergic rhinitis (AR) patients compared to healthy controls, showing that CD161+ T-cells expression was greatly associated with clinical severity. Thus, suggesting that CD161 expression may be involved in the pathogenesis of a given disease (118).

Since there is limited data regarding the effect of CMV and age on the expression of CD161 and CD300a receptors, and as CMV has a deep impact of the immune system and an important role in immunosenescence, the second objective of the present thesis was to study the effect of CMV latent infection and age on the expression of CD161 and CD300a receptors on CD4+, CD8+, NKT-like, and DN T-cell subsets, and their relation with the polyfunctionality marker CD57. This objective was addressed in the article "**Differential Effect of Cytomegalovirus Infection with Age on the Expression of CD57, CD300a, and CD161 on T-Cell Subpopulations**" Front Immunol. 2017 Jun 2;8:649. doi: 10.3389/fimmu.2017.00649. eCollection 2017.

Important TCR induced proteins for the differentiation of effector cytolytic T lymphocytes (CTLs) include the transcription factors T-bet and Eomes (119, 120). T-bet and Eomes share a sequence-specific T-box DNA-binding domain. They were identified for the first time in the murine Brachyury gene (121). Both transcription factors are known to be critically involved in developmental processes in vertebrates and have been shown to play an important role in immune cell development and cytolytic function. They are expressed in different human blood cell subsets, including CD4+, CD8+, γδ, iNK T-cells, NK cells, B cells, and dendritic cells (122). T-bet modulates certain genes involved in T-cell signaling Interleukin 12 receptor β1 (IL12Rβ1), cytolytic signaling molecules IFN-γ and cell mobilization CXC chemokine Receptor 3 (CXCR3) (123). Also, in murine models, T-bet was described as a key regulatory transcription factor involved in promoting Th1 CD4+ T-cell development, while inhibiting Th2 and Th17 lineages (124, 125). In addition, cytotoxic CD8+ T-cell effector differentiation and

up-regulation of perforin and granzyme B in antigen-specific cells are strongly related to high levels of T-bet expression (126, 127). Eomes is a novel Xenopus T-domain gene and plays an important role in regulating cytotoxic function, development, and survival of immune cells (128, 129). Eomes expression increases as cells become more memory-like (12, 130-132), whereas T-bet levels decrease (132). Several studies have reported that the process of memory formation is tightly regulated by T-bet and Eomes and that the differentiation of CD8+ effector T-cells is redundantly regulated by Eomes and T-bet (120, 130, 133). However, T-bet and Eomes were also found to have non redundant functions in determining the fate of CD8+ T-cells (130, 131). Thus, both transcription factors are important for the function and homeostasis of effector and memory T-cells. However, studies of T-bet and Eomes expression in the context of human T-cells are relatively limited and there is not sufficient information regarding the effects of ageing and CMV infection on T-bet/Eomes expression.

Furthermore, our previous results showed that CMV affects the phenotype and function of different T-cells subsets, in this regards we wanted to investigate the effect of CMV at molecular level. Additionally, as T-bet and Eomes had not been studied in regards of the polyfunctionality marker CD57 on T-cells, the third objective of the present thesis was to analyze the expression of the transcription factors T-bet and Eomes and CD57 marker on CD4+, CD8+ and $CD4^{hi}CD8^{lo}$ T-cell subsets in healthy individuals in the context of ageing and CMV infection. This objective was addressed in the article **" Effect of Cytomegalovirus (CMV) and Ageing on T-Bet and Eomes Expression on T-Cell Subsets "** *Int. J. Mol. Sci.***2017**, *18*(7), 1391; doi:10.3390/ijms18071391

3. HYPOTHESIS and OBJECTIVES

Aging and/or CMV infection are associated with changes in the phenotype and function of cytotoxic T lymphocyte cells.

The objectives of the present Doctoral Thesis are presented below:

Objective 1:

To study how age and CMV latent infection affect the frequency of NKT-like cells (CD8+CD56+ T cells) and their response to Staphylococcal Enterotoxin B (SEB) in the context of CMV and aging. *This objective was addressed in the article* ***"Effect of age and latent CMV infection on CD8+CD56+ T cells (NKT-like) frequency and functionality".*** *Mech Ageing Dev.2016 Sep;158:38-45. doi:10.1016/j.mad.2015.12.003. Epub 2016 Jan 2.*

Objective 2:

To analyze the effect of CMV-seropositivity and aging on the expression of CD300a and CD161 inhibitory receptors, along with the expression of CD57 marker on CD4+, CD8+, CD8+CD56+ (NKT-Like) and CD4−CD8− (DN) T-cell subsets. *This objective was addressed in the article "****Differential Effect of Cytomegalovirus Infection with Age on the Expression of CD57, CD300a, and CD161 on T-Cell Subpopulations****" Front Immunol.2017 Jun 2;8:649. doi: 10.3389/fimmu.2017.00649. eCollection 2017.*

Objective 3:

To analyze the expression of the transcription factors T-bet and Eomes and CD57 marker on CD4+, CD8+ and $CD4^{hi}CD8^{lo}$ T-cell subsets in healthy individuals, stratified by age and CMV serostatus. *This objective was addressed in the article* ***"Effect of Cytomegalovirus (CMV) and Ageing on T-Bet and Eomes Expression on T-Cell Subsets"****Int. J. Mol. Sci.****2017****, 18(7), 1391; doi:10.3390/ijms18071391*

4. PATIENTS, MATERIALS AND METHODS, RESULTS AND DISCUSSION

4.1. Effect of age and latent CMV infection on CD8+CD56+ T cells (NKT-like) frequency and functionality.

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Original article

Effect of age and latent CMV infection on CD8+ CD56+ T cells (NKT-like) frequency and functionality

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ABSTRACT

Changes in the T cell pool caused by CMV infection have been proposed to contribute to immunosenescence, but it has been postulated that CMV can also have some beneficial effects in young individuals improving the immune response to other pathogens. T cells expressing CD56 (NKT-like cells) are cytotoxic effector cells with a significant role in the immune response against cancer. We have studied how age and latent CMV infection affect the frequency of NKT-like cells (CD8+ CD56+ T cells) and their response to Staphylococcal Enterotoxin B (SEB) in the context of CMV and ageing. NKT-like cell percentage increases with the combination of both CMV and age. The response to SEB and the polyfunctional index of NKT-like cells also increase with age in CMV-seropositive individuals. In young individuals, CMV infection induces a shift on the polyfunctional profile of CD8+ CD56− T cells not observed on the NKT-like cells response. NKT-like cells expressing CD57 are expanded in CMV-seropositive individuals and are more polyfunctional than their CD57− counterpart. In addition CD57− NKT-like cells are more polyfunctional than CD8+ CD56− CD57− T cells. The results support that the expansion of polyfunctional NKT-cells may have a beneficial effect on the immune response against pathogens.

1. Introduction

The widespread herpesvirus *Cytomegalovirus* (CMV) has a prevalence that ranges between 60 and 70% in industrialized countries, reaching almost 100% in emerging countries (Fulop et al., 2013). It is associated with geographic, ethnic and social factors as well as with age (Cannon et al., 2010). The prevalence of CMV in Spain is very high and more than 80% of individuals over 40 years are CMV-seropositive (deOry et al., 2004). Although CMV is harmless for adult immunocompetent individuals, it can trigger severe disease in immunocompromised individuals (Fortun et al., 2010; Sissons and Wills 2015; Smithers-Sheedy et al., 2015). Infection by CMV associates with alterations of the immune system, playing a central role in the process of immunosenescence (Pawelec et al., 2005; Pawelec and Derhovanessian, 2011; Fulop et al., 2013; Pawelec, 2014).

A hallmark of human immunosenescence is the accumulation of memory T lymphocytes together with a reduction of naïve T cells. The major age-associated changes affect the CD8+ rather than the CD4+ T cell subset. This accumulation of memory CD8+ T cells, observed with age, is associated with CMV infection and not with other persistent herpesviruses (Almanzar et al., 2005; Chidrawar et al., 2009; Derhovanessian et al., 2011). Furthermore, seropositivity to CMV correlates with the immune risk profile (IRP), characterized by poor proliferative responses of T cells after polyclonal stimulation, accumulation of CD8+ CD28− T cells and an inversion in the CD4+/CD8+ T cell ratio (Wikby et al., 1998; Wikby et al., 2002). Several reports have shown that the age-associated expansion of CD8+ CD28− T cells occurs during CMV infection (Wang et al., 1995; Hooper et al., 1999; Khan et al., 2002; Wikby et al., 2002). These cells have a diminished proliferative and antiviral activity (Appay et al., 2002). Moreover, CMV infection has been correlated with a reduced response to influenza vaccination (Trzonkowski et al., 2003; Derhovanessian et al., 2014; Frasca et al., 2015) and higher risk of influenza complications in the elderly (Moro-Garcia et al., 2013). CMV-seropositivity is also associated with accelerated T cell immunosenescence and stronger pro-inflammatory response after acute myocardial infarction and

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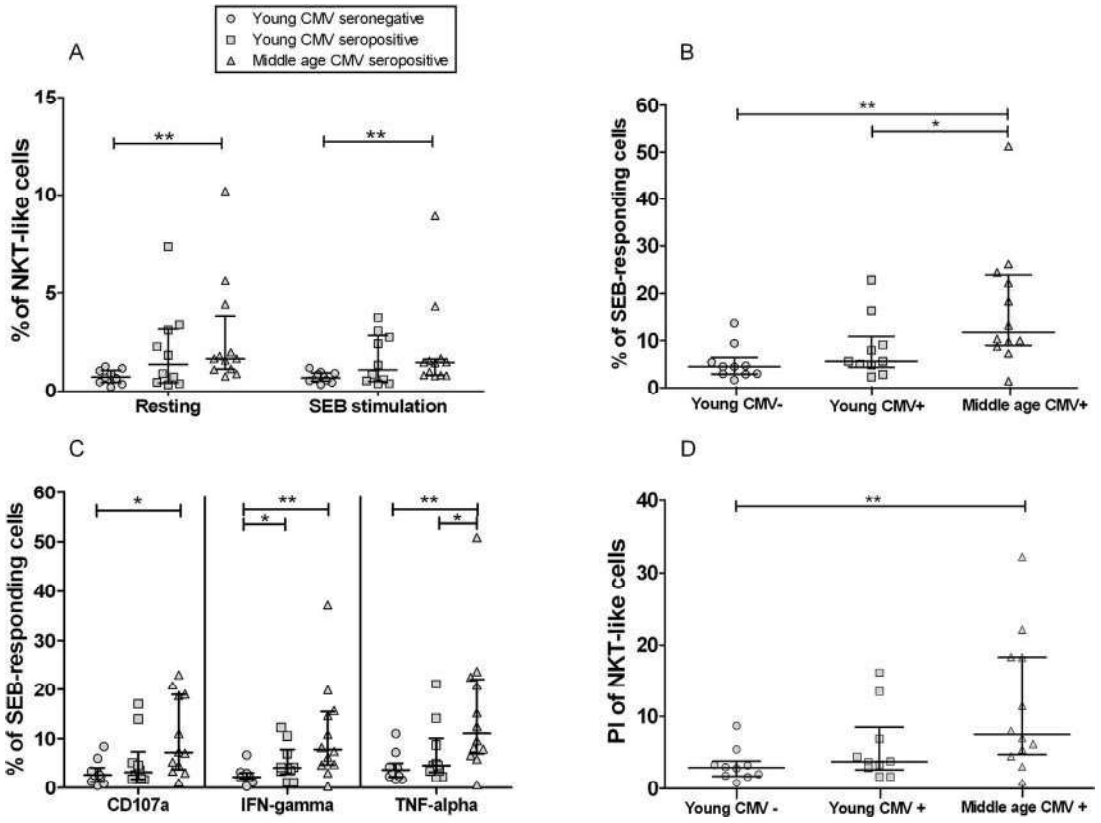

Fig. 1. Percentage and SEB-responses of NKT-like cells. (A) Scatter graph shows NKT-like cells (CD8+ CD56+ T cells) from young (CMV-seronegative and seropositive) and middle age CMV-seropositive individuals, resting and stimulated with SEB. (B) Percentage of NKT-like cells that have any studied response to SEB. (C) Individual SEB-responses of NKT-like lymphocytes. (D) Polyfunctional index (PI) of NKT-like cells in the three groups studied. Vertical black lines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line.

reperfusion (Shmeleva et al., 2015; Hoffmann et al., 2015). Therefore, the alterations in the T cell pool caused by CMV infection have been proposed to contribute to early onset of immunosenescence (Derhovanessian et al., 2009).

Despite all these data suggesting a detrimental role of CMV over the immune system, recent controversial results have raised a discrepancy. In a murine model, it has been demonstrated that increased levels of IFN-gamma due to murine CMV (MCMV) are protective against other pathogens and MCMV-seropositive mice exposed to bacterial pathogens were resistant to infection, while seronegative mice were not (Barton et al., 2007). It has been recently demonstrated that CMV-seropositive young adults have an enhanced antibody response to influenza vaccination compared to seronegative individuals (Furman et al., 2015). In a similar way young mice infected with murine CMV also show significant protection from an influenza virus challenge compared with uninfected animals (Furman et al., 2015). Our group has also shown an increase of CD8+ T cell polyfunctionality in young individuals seropositive for CMV due to the expansion of CD8+ CD57+ T cells, supporting the hypothesis that latent infection by herpesviruses, like CMV, improve the quality of the immune response to other pathogens (Pera et al., 2014). These results raise the question of whether the CMV infection is harmful and/or beneficial for the immune system depending on the age of the individuals studied.

Both CMV infection and ageing also associate with increased expression of CD56 on T cells in humans (Looney et al., 1999; Tarazona et al., 2000; Peralbo et al., 2007; Almehmadi et al., 2014). CD56 expressing T cells are often referred as Natural killer T (NKT) cells or NKT-like cells to avoid confusion with invariant NKT (iNKT) cells that are CD1d-restricted and express a semi-invariant TCR-alpha chain (Peralbo et al., 2007). iNKT cells are a minor subset of peripheral blood (<0.01%), considered to be regulatory cells that produce cytokines (IL-4 and IFN-gamma) in response to glycolipids, and their frequency decrease with ageing (Tarazona et al., 2000; DelaRosa et al., 2002; Peralbo et al., 2006). On the contrary, CD8+ CD56+ (NKT-like) T cells represent 1–11% of the peripheral T cell pool, are CD1d-unrestricted T cells with an oligoclonal TCR that recognises peptides bound to classic HLA class I molecules, display an effector memory or effector phenotype and have high tumour cytotoxic capacity and cytokine production (Tarazona et al., 2000; Kelly-Rogers et al., 2006). Whereas NKT-like cells can contribute to cancer immunosurveillace (Alves et al., 2011; Zdrazilova-Dubska et al., 2012), it has also been postulated that they can be involved in tissue damage after acute myocardial infarction (Romo et al., 2011; Shmeleva et al., 2015; Hoffmann et al., 2015) or chronic pulmonary diseases (Tang et al., 2013; Papakosta et al., 2014; Hodge et al., 2015).

It has been established that iNKT cells play an important role in the immune response against infections (Juno et al., 2012), but little is known about the contribution of NKT-like cells. A higher proportion of these cells producing high levels of inflammatory cytokines and CD107a were shown in CMV-seropositive than in CMV-seronegative healthy subjects (Almehmadi et al., 2014).

There are very few studies regarding the effect of age and CMV infection on NKT-like cell responses to pathogens. Thus, here we propose the analysis of NKT-like cells responses to Staphylococcal Enterotoxin B (SEB), in the context of latent CMV infection and ageing. In particular we analyse the functional capacities of NKT-like cells –degranulation (CD107a) and/or cytokines co-production (INF-gamma and TNF-alpha)– in comparison with CD8+ CD56– T cell functionality.

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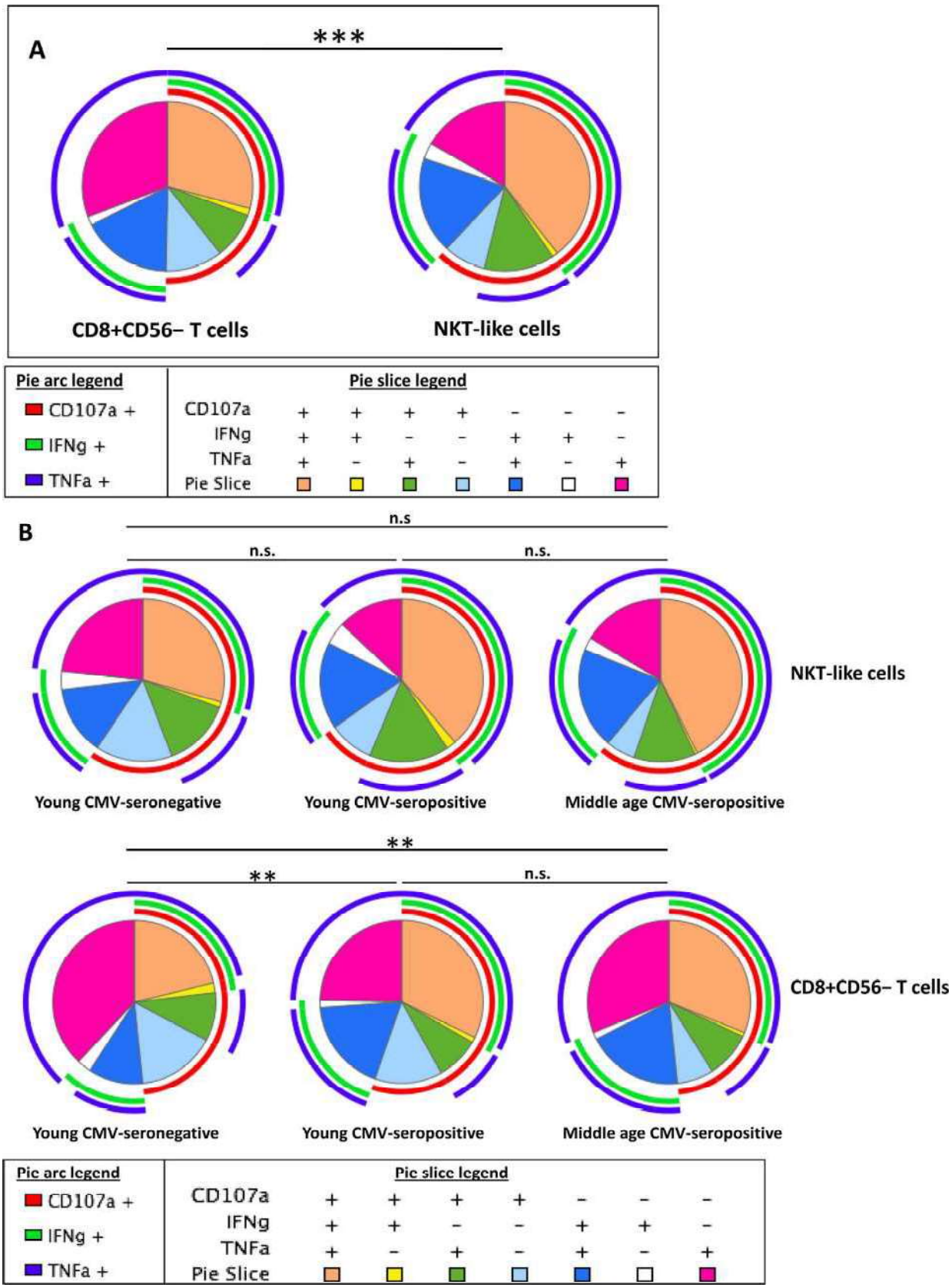

Fig. 2. SEB-induced polyfunctional profiles. (A) SPICE polyfunctional profiles of NKT-like and CD8+ CD56− T cell subsets from all individuals ($n = 32$). (B) Polyfunctional profile stratification by age and CMV serostatus of NKT-like cells and CD8+ CD56− T cells. Each functional category (pie slice) is described in Table below at the bottom.

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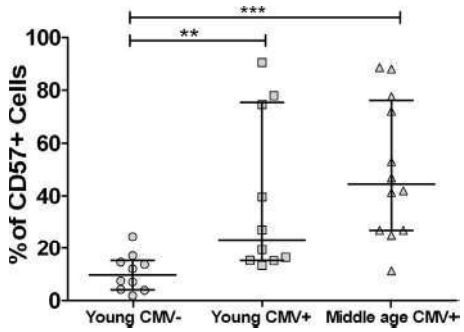

Fig. 3. Expression of CD57 by NKT-like Percentage of CD57+ NKT-like cells in the three groups stratified by age and CMV serostatus.

Our results show that NKT-like cells are increased in middle age CMV-seropositive donors. The response to SEB and the polyfunctional index (PI) of NKT-like cells also increase with age in CMV-seropositive individuals. NKT-like cells have a different polyfunctional profile than CD8+ CD56− T cells and, in contrast to CD8+ CD56− T cells, it is not affected by CMV infection in young individuals.

2. Material and methods

2.1. Subjects

In order to study SEB-responses of NKT-like cells we used 32 healthy individuals stratified in three groups according to their age (young and middle age) and CMV serostatus as described in Supplemental Table S1. Individuals in the middle age group were CMV-seropositive as we were not able to recruit enough CMV-seronegative individuals due to the high prevalence of CMV in Spain in this age range, over 80% in older than 40 years old (deOry et al., 2004). All subjects studied met the following exclusion criteria: diabetes, cancer, renal failure, liver diseases, endocrine disorders, autoimmune diseases, or acute infectious disease; and were not consuming drugs whose activity is known to modify the functions of the immune system. The study was approved by the Ethics Committee of the Reina Sofia University Hospital. All donors participating in the study provided informed written consent.

2.2. Stimulation, intracellular staining, and detection of CD107a expression

All experiments were conducted as previously described in detail (Pera et al., 2014). Briefly, PBMCs were cryopreserved and after thawing, maintained overnight in a standard incubator (humidified CO_2 atmosphere) at 37 °C. Cells were then placed in a 96 well plate at 2 × 106 cells/ml concentration (250 μl final volume). Anti-CD107a-APC (BD Biosciences) was added to all wells. For stimulation 1 μg/ml of Staphylococcal Enterotoxin B superantigen (SEB, Sigma–Aldrich) was added together with costimulatory antibodies (anti-CD28 and anti-CD49d, 1 μg/ml each; BD Biosciences). Cells were incubated 5 h at 37 °C (Golgistop, 0.67 μl/ml and Golgi Plug, 1 μg/ml; BD Biosciences were added after one hour of incubation). Following stimulation, cells were washed with PBS (4 °C) and stained with surface antibodies (CD57-VioBlue, Miltenyi Biotec and CD56-PE-Cy7, BD Biosciences). For intracellular staining with CD3-PerCP, CD8-APC-Cy7 (BD Biosciences), IFN-gamma-FITC and TNF-alpha-PE (Miltenyi Biotec) antibodies, cells were first fixed and permeabilized with Cytofix/Cytoperm solution according to the manufacturer's instructions (BD PharMingen). We used the same protocol for isotype controls. All antibodies were titrated before use. Cells were analysed by flow cytometry the following day.

2.3. Flow cytometry and data analysis

Samples were acquired with a 9 parameters MACsQuant instrument (Miltenyi Biotech) and analysed with FlowJo v X 10.0.7 software (TreeStar, Portland OR). We collected 30.000–50.000 events of CD3+ CD8+ gate for each sample. All files were gated on small lymphocytes (forward *vs* side scatter). For CD57 expression assessment we gated on resting CD8+ CD56− T cells or CD8+ CD56+ T cells (NKT-like cells).

Functionality including degranulation and cytokine-production was analysed measuring CD107a, IFN-gamma or TNF-alpha events gated on CD3+ CD8+ CD56− T cells or CD3+ CD8+ CD56+ T cells. Polyfunctionality gating strategy is illustrated in Supplemental Fig. S1. To study SEB-induced polyfunctionality of CD8+ CD56− or NKT-like cells, according to the expression of CD57 marker, CD3+ and subsequently CD8+ CD56− or CD8+ CD56+ (NKT-like) cells were gated and then divided into the following quadrants: CD57− CD107−, CD57− CD107+, CD57+ CD107− and CD57+ CD107+. IFN-gamma and/or TNF-alpha were then gated on each of these subsets. Settings for the three functions studied (CD107a, IFN-gamma and TNF-alpha) were defined on the basis of a negative control (unstimulated cells).

Analysis of polyfunctional data was performed by SPICE 5.35 software (Mario Roederer, ImmunoTechnology Section, Vaccine Research Centre, NIH, Bethesda, MD) (Roederer et al., 2011). The polyfunctional index (PI) was calculated as described (Larsen et al., 2012) by using the Funky Cells Toolbox (http://www.funkycells.com/main/).

2.4. Statistical analysis

Net responses were obtained after subtracting the negative control values from the SEB responses. To assess whether the data followed normal distribution we used the Kolmogorov–Smirnov test. Since no normality was found, Mann–Whitney U nonparametric test was used for comparing data among each sample pairs. To compare the pie charts we used SPICE's permutation analysis, which asks how often, given the samples that comprise the two pie charts, the difference observed would happen simply by chance (10,000 permutations). All statistical tests were performed with PASW Statistics v18; p values < 0.05 were considered significant.

3. Results

3.1. Age and CMV seropositivity impact on NKT-like cell frequency and SEB-induced responses

Our results indicated that the percentage of CD8+ CD56+ T cells (NKT-like) of middle age CMV-seropositive subjects was higher than in young CMV-seronegative both in resting and SEB-stimulated cells, whereas no significant differences were observed in young subjects according to CMV serostatus (Fig. 1A). Although the absolute counts of NKT-like T cells increased in the middle age CMV-seropositive group no statistically significant differences were found, likely due to the high variability observed (data not shown).

SEB-induced responses of NKT-like cells were evaluated by flow cytometry measuring simultaneously: IFN-gamma, TNF-alpha, and CD107a. Gating strategy used is shown in Fig. S1. We observed that the number of NKT-like cells responding to SEB, with any of the studied responses, was higher in middle age CMV-seropositive donors than in the young (CMV-seropositive and CMV-seronegative). We did not observe significant differences between young CMV-seronegative and CMV-seropositive subjects (Fig. 1B). These results are similar to those previously reported for overall CD8+ T cells (Pera et al., 2014).

As shown in Fig. 1C, when each function was analysed separately we observed an increase in IFN-gamma producing NKT-like cells in young CMV-seropositive donors compared with CMV-seronegative, whereas there was an age-associated increase in NKT-like cells producing TNF-alpha in CMV-seropositive donors. Besides, age and CMV showed an additive effect increasing CD107a expression (middle age CMV-seropositive donors *vs* young CMV-seronegative). However, the response of NKT-like cells to SEB was lower than CD8+ CD56− T cells. After stratifying the cohort by age and CMV serostatus this difference was only found in the young groups and not in the middle age group (data not shown).

Finally to assess the effect of age and CMV infection on NKT-like cell SEB-induced polyfunctional index (PI) (Larsen et al., 2012) we performed a comparison among the three groups. In that regard, PI of NKT-like cells increased with age being significantly different only among the young CMV-seronegative and middle age CMV-seropositive groups (Fig. 1D).

3.2. Polyfunctional profiles of NKT-like cells and CD8+ CD56− T cells in response to SEB

We further used SPICE permutation analysis (Roederer et al., 2011) to compare the polyfunctional profiles of NKT-like and CD8+ CD56− T cells. Polyfunctional profiles of both subsets resulted different (Fig. 2A) even splitting the samples by age and CMV status (data not shown). In addition, we studied the polyfunctional

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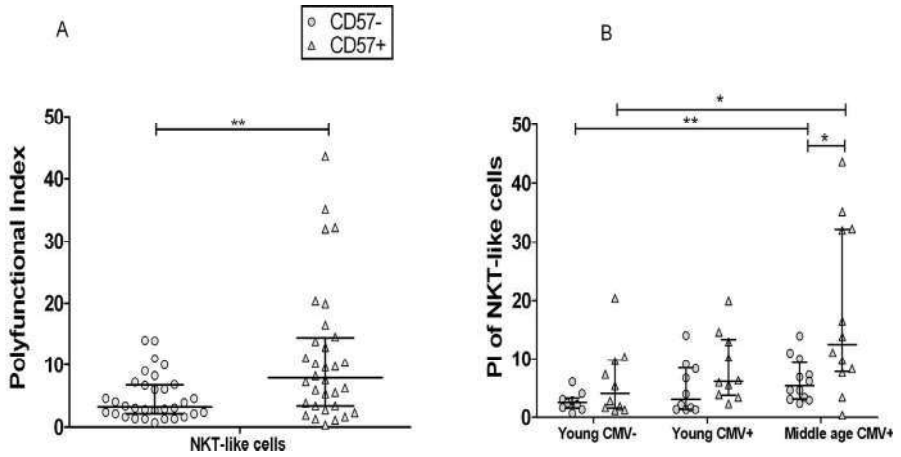

Fig. 4. Expression of CD57 by NKT-like and functional comparison of CD57+ and CD57− NKT-like subsets (A) PI of NKT-like cells according CD57 expression in all individuals ($n = 32$). (B) PI of NKT-like cells according CD57 expression, stratifying the sample by age and CMV serostatus. Vertical black lines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line.

profiles of both T cell subsets among the groups. The polyfunctional profile of NKT-like cells was not affected by age or CMV infection whereas differences were observed according to CMV serostatus, but not according to age, in the CD8+ CD56− T cell population (Fig. 2B). Thus, CMV induces a shift on the SEB-induced functional profile of CD8+ CD56− T cells in young subjects, but not on NKT-like cells.

3.3. *Expansion CD8+ CD57+ T cells is a hallmark of latent CMV infection and their polyfunctionality is unrelated to CD56 expression*

Our previous results demonstrate that CD8+ CD57+ T cells display higher polyfunctionality than their CD8+ CD57− counterpart (Pera et al., 2014). Additionally, CD8+ CD57+ T cell expansion was associated with an improvement of CD8+ T cell SEB-response in young individuals. Therefore, we studied NKT-like cell functionality according to CD57 expression.

We determined CD57 expression on unstimulated CD8+ CD56+ T cells. The results indicated that CD57+ NKT-like cell percentage was higher in CMV-seropositive donors (young and middle age) compared to CMV-seronegative young donors (Fig. 3). Moreover, CD57 expression was higher in NKT-like cells than in CD8+ CD56− T cells independently of age and CMV serostatus (young CMV-seronegative $p = 0.001$, young CMV-seropositive $p = 0.049$ and middle age $p = 0.001$).

The analysis of the PI of NKT-like cells according to CD57 expression showed that cells expressing CD57 had higher PI than CD57 negative cells (Fig. 4A). However, the PI of CD57+ NKT-like T cells was higher than the PI of their CD57− counterpart only in the middle age CMV-seropositive individuals and not in the young. In addition, the PI of NKT-like cells was increased in middle age CMV-seropositive individuals compared with young CMV-seronegative independently of CD57 expression (Fig. 4B).

We also analysed SEB-induced polyfunctional profiles of NKT-like and CD8+ CD56− T cells according to CD57 expression using the SPICE software. The results obtained indicated that CD8+ CD57− and CD8+ CD57+ T cells have different profiles independently of CD56 expression (Fig. 5). Nevertheless, when the analysis was performed stratifying the sample by age and CMV status, the difference between CD57− and CD57+ NKT-like subsets was only observed in the young CMV-seropositive individuals (data not shown). Additionally, polyfunctional profiles of NKT-like *vs* CD8+ CD56− T cells negative for CD57, were also statistically different, while the polyfunctional profiles of CD57+ cells did not change with CD56 expression (Fig. 5).

4. Discussion

It has been established that NKT-like cells expand with age (Looney et al., 1999; Tarazona et al., 2000; Peralbo et al., 2007; Almehmadi et al., 2014) and it has also been suggested that this cell subset increases with CMV infection in healthy and coronary disease patients (Romo et al., 2011; Bergstrom et al., 2012; Shmeleva et al., 2015). However, the cohort studied by Bergstrom et al. (2012) included only old individuals (>60 years) and in the stratification performed by Almehmadi et al. (2014) the age of the young group ranged from 23 to 60 years old. In the groups of coronary patients the age range was 34–87 years old (Romo et al., 2011) or between 54 (percentile 25) and 72 (percentile 75) (Shmeleva et al., 2015) and they did not stratified by age. Therefore, this is the first time that the frequency of NKT-like cells (CD8+ CD56+ T cells) is analysed in young healthy individuals (18–35 years) stratified by CMV serostatus. Opposite to the results obtained in older cohorts, in our sample of young individuals, NKT-like cell frequency did not change between CMV-seronegative and CMV-seropositive individuals. We do not observe any change until CMV-seropositive individuals reach older ages, suggesting that NKT-like cells accumulate in CMV-seropositive individuals with age, rather than with CMV infection per se.

The response of NKT-like cells (CD8+ CD56+ T cells) to SEB has not been studied before. The increase of NKT-like cells percentage in CMV-seropositive subjects observed in middle age is accompanied by an increase in their response to the bacterial toxin SEB. In addition, our results show an increase of NKT-like cells' PI with age in CMV-seropositive individuals. Thus, NKT-like cell functionality in response to SEB increases quantitatively and qualitatively with age in CMV-seropositive individuals.

As we reported previously (Pera et al., 2014), overall CD8+ T cells SEB-response was also increased in middle age CMV-seropositive donors compared to young. Thus, independently of CD56 expression, the response of CD8+ T cells to SEB increases with age in CMV-seropositive individuals. However, CD8+ CD56− T cells display a stronger response to SEB than NKT-like cells. Particularly, there is an increase of cytokine producing cells in the CD8+ CD56− subset. SEB causes a toxic shock in humans and in sensitized mice that can be lethal. This toxin acts as a superantigen leading to excessive T cell activation and cytokine production. Cytokines produced by T cells in response to SEB contribute to the development of lethal

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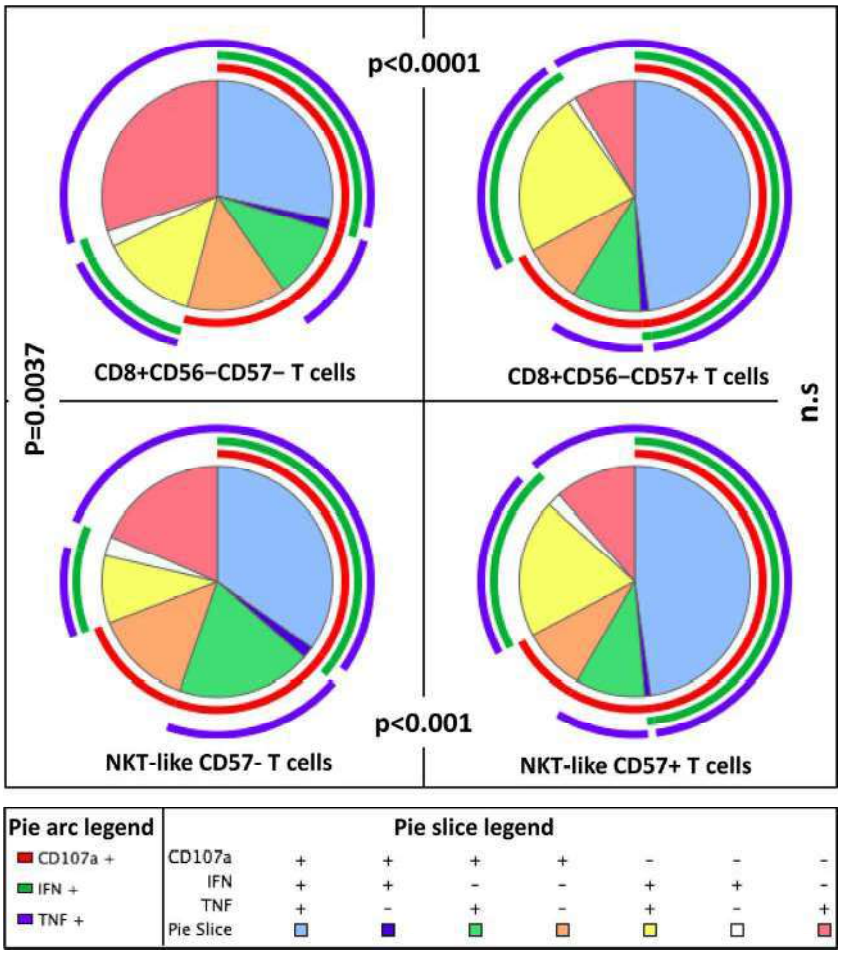

Fig. 5. SEB-polyfunctional profiles of NKT-like and CD8+ CD56− T cells according to CD57 expression. Pie charts corresponding to polyfunctional profiles of CD8+ CD56− or CD8+ CD56+/CD57− or CD57+ T cell subsets in all individuals studied ($n = 32$). The combination of functions studied (pie slices) is indicated in Table below the pie charts.

toxic shock, in particular TNF-alpha production (Miethke et al., 1992). Our results regarding SEB-response have shown that NKT-like cells have a lower response to SEB than CD8+ CD56− T cells, and particularly a lower frequency of TNF-alpha mono-functional cells, suggesting that NKT-like cells may not have a relevant role in the toxic shock induced by SEB toxin.

CMV infection has no effect on NKT-like cell subset functionality in response to SEB whereas it increases the functionality of CD8+ CD56− T cells in young individuals, as we previously demonstrated for overall CD8+ T cells (Pera et al., 2014). This difference could be due to the low response of NKT-like cells to SEB and/or to the low number of these cells in young donors. Besides, NKT-like and CD8+ CD56− T cells differ in their polyfunctional profiles.

Most T cells expressing CD57 lack CD28 and have been traditionally described as terminally differentiated cells that are senescent and/or dysfunctional (for review see Weng et al. (2009); Chou and Effros (2013)). However, our recently published results diverge from that vision and suggest that CD8+ T cells expressing CD57 are, at least in healthy donors (young and middle age), polyfunctional T cells that could be implicated in heterologous immunity (Pera et al., 2014). This polyfunctional cell subset expands with latent CMV infection rather than with age per se. Here we also report the functional characterization of NKT-like cells according to CD57 expression. CD57+ NKT-like cells expand mainly with CMV infection. Besides, in middle age subjects, there is a higher proportion of cells expressing CD57 in the NKT-like cell subset compared to CD8+ CD56− T cells.

We also confirmed that, independently of CD56 expression, CD8+ CD57+ T cells respond differently to SEB than its CD8+ CD57− counterpart, due to a higher polyfunctionality of CD8+ CD57+ T cells. This suggests that the increase of NKT-like cells polyfunctionality in the middle age group is mostly caused by the accumulation of polyfunctional cells that express CD57.

It has been established that ageing is accompanied by systemic inflammation, what has been called “inflamm-ageing”. One biomarker of inflamm-ageing is TNF-alpha. Thus, our results indicate that CD8+ CD56− T cells contribute to this chronic inflammation process. Inflamm-ageing has been implicated in the pathogenesis of several of the major age-related diseases (cardiovascular disease, type 2 diabetes and dementia) and has been associated with increased mortality (Franceschi et al., 2000; Salvioli et al., 2013; Franceschi and Campisi 2014). In addition, CMV seropositivity together with higher CRP correlate with all-cause mortality (Simanek et al., 2011) although recent results indicate that CMV is not a primary cause of age-related systemic inflammation (Bartlett et al., 2012)

In conclusion, the results presented demonstrate that CD57 is a polyfunctional marker for CD8+ T cells including CD8+ CD56+ NKT-like cells that expand mainly with CMV infection. In CMV-seropositive individuals, with age, NKT-like subset expands and is enriched in SEB-responding polyfunctional CD57+ cells. Moreover, CD8+ CD56− T cells response to SEB, in particular the cytokine production, increases with age. In contrast to the classical negative effect described for CMV infection in old ages, our results support the hypothesis that CMV infection in younger ages could

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have a protective role by promoting heterologous immunity. Heterologous immunity refers to the fact that immunity to a previously encountered pathogen can alter the immune response against other unrelated pathogens (Sharma and Thomas, 2014). It is commonly observed in persistently infected individuals who experience chronic antigenic stimulation (such as herpes viruses, including CMV) that alters their immunity to other pathogens (Barton et al., 2007). Most data regarding heterologous immunity have been obtained using murine models, however, there is also evidence of this phenomenon in human (Welsh et al., 2010; Sharma and Thomas, 2014; Che et al., 2015). Moreover, Miles et al. (2008) studied the effect of CMV infection on T cell response to measles vaccination in infants, and they observed a correlation between the antibody response to measles and the IFN-gamma response to CMV, as well as an increased SEB-induced CD8+ T cell proliferation in CMV-seropositive infants, suggesting that CMV infection confers protection against some pathogens in infancy by enhancing some immune responses. Besides, a recent report has shown that latent CMV infection enhances the response to influenza vaccination in young individuals, while age associates with decreased responses to vaccination regardless of CMV status (Furman et al., 2015). CMV-seropositive young individuals had better antibody responses to influenza vaccination, higher CD8+ T cell sensitivity, as well as increased levels of circulating IFN-gamma than CMV-seronegative individuals.

Our results and these reports support the hypothesis that CMV has a beneficial role on the immune response in early stages of life. More studies regarding all these questions should be addressed and CMV serostatus determination should be considered in all studies addressed to analyse the immune response in different clinical situations in particular in those associated with ageing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2015.12.003.

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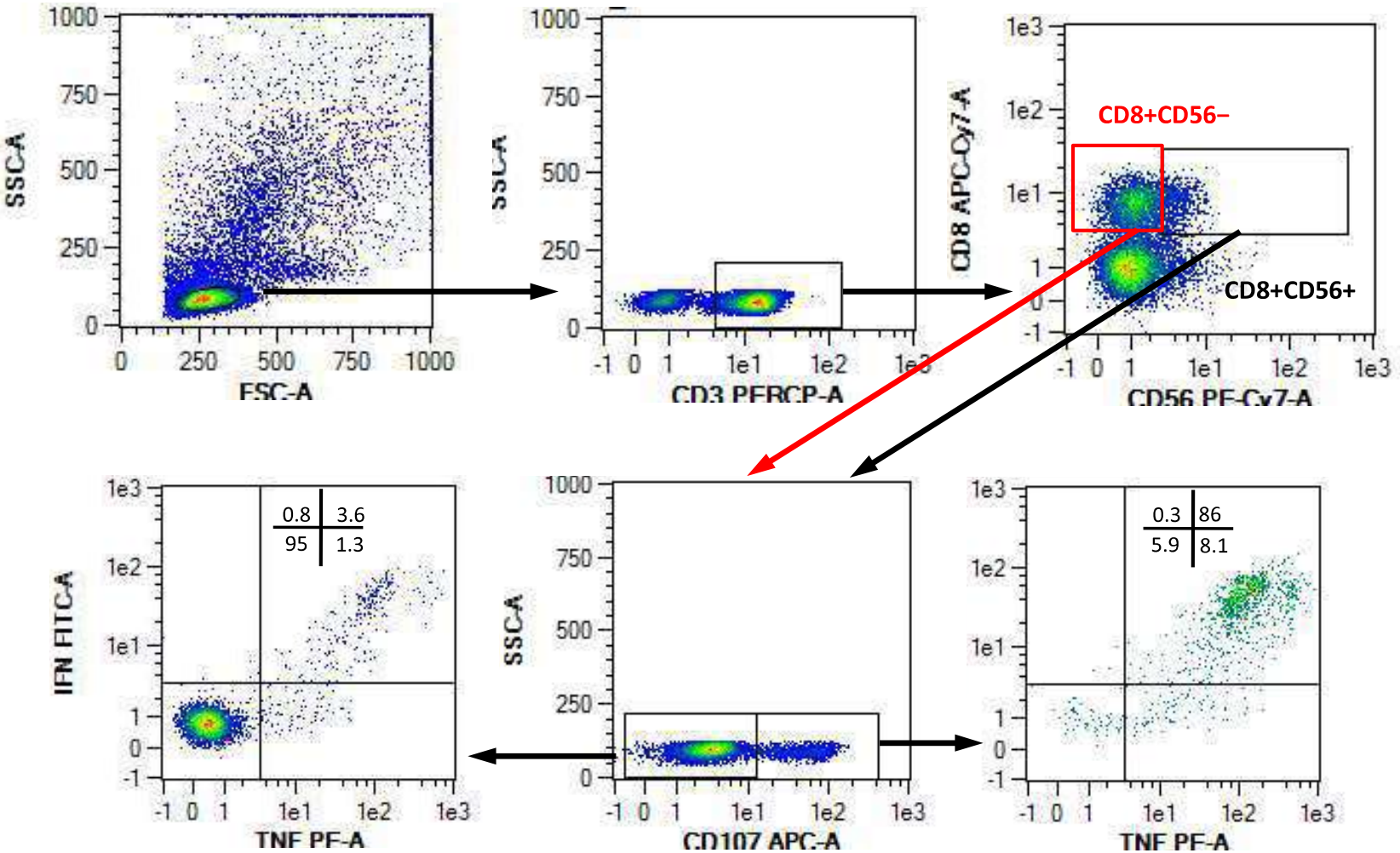

Figure S1. Flow cytometry gating strategy used in the analysis of polyfunctionality. Figure shows PBMCs from a CMV-seropositive young healthy individual, stimulated with SEB. After initial gating on lymphocytes, cells were then selected based on CD3+ staining and subsequent CD8+CD56− or CD8+CD56+ (NKT-like) coexpression and then divided into two gates CD107− and CD107+. Cytokine production was measured in the CD107 gates by confronting IFN-gamma and TNF-alpha (IFNg−TNFa−, IFNg+TNFa−, IFNg−TNFa+, IFNg+TNFa+). Values are referred to CD8+CD56− or NKT-like cells.

Table S1. Demographic characteristics of the groups studied.

	Young CMV-seronegative	Young CMV-seropositive	Middle age CMV-seropositive
Age range (years)	18-35	18-35	40-60
Age median (years)	26.3, SD=5.8	27, SD=4.3	50.25, SD=4.3
Male/female	4/6	6/4	3/9

4.2. Differential Effect of Cytomegalovirus Infection with Age on the Expression of CD57, CD300a, and CD161 on T-Cell Subpopulations

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Differential Effect of Cytomegalovirus Infection with Age on the Expression of CD57, CD300a, and CD161 on T-Cell Subpopulations

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Immunosenescence is a progressive deterioration of the immune system with aging. It affects both innate and adaptive immunity limiting the response to pathogens and to vaccines. As chronic cytomegalovirus (CMV) infection is probably one of the major driving forces of immunosenescence, and its persistent infection results in functional and phenotypic changes to the T-cell repertoire, the aim of this study was to analyze the effect of CMV-seropositivity and aging on the expression of CD300a and CD161 inhibitory receptors, along with the expression of CD57 marker on $CD4^+$, $CD8^+$, $CD8^+CD56^+$ (NKT-Like) and $CD4^-CD8^-$ (DN) T-cell subsets. Our results showed that, regardless of the T-cell subset, $CD57^-CD161^-CD300a^+$ T-cells expand with age in CMV-seropositive individuals, whereas $CD57^-CD161^+CD300a^+$ T-cells decrease. Similarly, $CD57^+CD161^-CD300a^+$ T-cells expand with age in CMV-seropositive individuals in all subsets except in DN cells and $CD57^-CD161^+CD300a^-$ T-cells decrease in all T-cell subsets except in $CD4^+$ T-cells. Besides, in young individuals, CMV latent infection associates with the expansion of $CD57^+CD161^-CD300a^+CD4^+$, $CD57^-CD161^-CD300a^+CD4^+$, $CD57^+CD161^-CD300a^+CD8^+$, $CD57^-CD161^-CD300a^+CD8^+$, $CD57^+CD161^-CD300a^+$ NKT-like, and $CD57^+CD161^-CD300a^+$DN T-cells. Moreover, in young individuals, CD161 expression on T-cells is not affected by CMV infection. Changes of CD161 expression were only associated with age in the context of CMV latent infection. Besides, $CD300a^+CD57^+CD161^+$ and $CD300a^-CD57^+CD161^+$ phenotypes were not found in any of the T-cell subsets studied except in the DN subpopulation, indicating that in the majority of T-cells, CD161 and CD57 do not co-express. Thus, our results show that CMV latent infection impact on the immune system depends on the age of the individual, highlighting the importance of including CMV serology in any study regarding immunosenescence.

Keywords: CD57, CD300a, CD161, T-cell subsets, age and cytomegalovirus infection

INTRODUCTION

The human CD300 family has seven members, including the inhibitory receptor CD300a, which has been proposed as a possible biomarker for diagnosis and therapeutic target in several pathological situations (i.e., infectious diseases and cancer) (1–4). Human CD300a receptor is expressed on the surface of T (5, 6) and natural killer (NK) cells (7, 8). On human NK cells, the interaction between

CD300a and its ligand reduces their cytotoxic function (8). In T and B cells, the primary function of CD300a is to limit antigen receptor-mediated positive signaling (9). However, on $CD8^+$ T-cells, CD300a expression has been shown to associate with better cytotoxic function (10) and $CD300a^+CD4^+$ T-cells are associated with polyfunctionality and, upon stimulation, upregulate the transcription factor Eomesodermin (Eomes) (6, 11).

CD161 marker is a C-type lectin that was originally described in NK cells (12, 13). Nevertheless, CD161 is also expressed by T-cells including $CD4^+$, $CD8^+$ (12), and γδ T-cells (14). Within the $CD4^+$ subset, CD161 expression has been associated with IL-17 production. Indeed, Th17 cells can be originated from the $CD161^+CD4^+$ but not from their $CD161^-CD4^+$ counterpart (15). Of note, other IL-17-producing T-cells, such as $CD8^+$ and $CD4^-CD8^-$ double-negative T-cells are as well $CD161^+$ (16). Furthermore, it has been shown that CD161 expression on T-cells characterizes a transcriptional and functional T-cell phenotype that is TCR- and cell lineage-independent (17). All $CD161^+$ T-cell subsets shared a transcriptional signature and responded in a TCR-independent (innate-like) way to cytokine stimulation (IL-12 plus IL-18). However, CD161 had no regulatory effect on this response. Instead, CD161 has been shown to function as a costimulatory receptor in the context of TCR stimulation (18, 19). While the role of CD161 receptor on NK cells is agreed to be inhibitory (12, 20, 21), on T-cells, there is lack of consensus, as there is evidence of both costimulatory (20, 22) and inhibitory (18, 19) effects.

During aging, both innate and adaptive immunity are affected. Age-related changes have been described in several immune cell types including T-cells, NK cells, B-cells, macrophages, etc. Among those changes, the alterations in the number, phenotype, and functional capacity of immune cells have been associated with higher susceptibility to infectious diseases that ultimately lead to increased risk of fragility and death in those individuals (23–26). This age-associated deterioration of the immune system has been termed "immunosenescence." However, immunosenescence is not exclusively due to chronological aging of the individual and there are situations involving chronic stimulation of the immune system, such as viral infections, in which an "immunosenescence accelerated" or "early immunosenescence" is observed (27–30). In humans, infection by a common virus, cytomegalovirus (HCMV) has been shown to have profound impact on the T-cell compartment both on $CD8^+$ and $CD4^+$ T-cells (31, 32). HCMV persists after primary infection and is continuously controlled by the immune system (33, 34). Human herpes viruses, like CMV, have generally a benign/symbiotic relationship with the host (35–38). However, this benign relationship between herpesviruses and its hosts is altered with age. Indeed, CMV latent infection has been related to early immunosenescence (32, 39, 40). Particularly, CMV-seropositivity is associated with an increased risk of death and cardiovascular diseases (41–43) and is a contributor to the development of an "Immune Risk Phenotype" (IRP). This IRP is associated with early mortality in the elderly (44–46). Therefore, HCMV is considered one of the most relevant contributors to immunosenescence.

Thus, both HCMV infection and age contribute to the process of immunosenescence inducing changes on the T-cells. Understanding the mechanisms leading to immunosenescence and finding new biomarkers could open the possibility of novel therapies for the treatment of age-related diseases. In that regard, here, we study the effect of CMV latent infection and age on the expression of CD161 and CD300a receptors on $CD4^+$, $CD8^+$, $CD8^+CD56^+$ (NKT-like), and $CD4^-CD8^-$ (DN) T-cell subsets and their relation with the polyfunctionality marker CD57, which is a hallmark of CMV infection and aging in T-cells (37, 38).

MATERIALS AND METHODS

Subjects

We studied 64 healthy individuals stratified according to age and CMV serostatus (**Table 1**). Individuals in the old group and middle age group were all CMV-seropositive, as we were not able to recruit enough CMV-seronegative individuals due to the high prevalence of CMV seropositivity in Spain, which is about 80% in individuals over the age of 40 years (47) and reaches about 99% in individuals over 65 years in Andalusia (Southern Spain) where the samples were collected.

All subjects studied met the following exclusion criteria: absence of diabetes, cancer, severe renal failure, severe liver disease, endocrine disorders, autoimmune diseases, or acute infectious disease; they were not consuming drugs whose activity is known to modify the functions of the immune system. Ethical statement was approved by the Ethics Committee of the Reina Sofia University Hospital and all study participants provided informed written consent.

CMV Serology

CMV-specific IgG and IgM was determined in sera by using automated enzyme-linked immunosorbent assay (ELISA) (DRG International, Mountainside, NY, USA).

Flow Cytometry and Data Analysis

Peripheral blood from each subject was collected in lithium heparin tubes, followed by PBMCs isolation by density gradient centrifugation using Ficoll Histopage-1077 (Sigma-Aldrich, St. Louis, MO, USA). After isolation, PBMCs were cryopreserved until experiments were performed.

Cell thawing was carried out in RPMI 1640 (Sigma-Aldrich) with 10% FBS (Gibco, Life Technologies CA, USA) and cells were placed in a 96-well plate at 2×10^6 cells/ml concentration (250 µl final volume). Subsequently, cells were washed twice with PBS (4°C) and stained for the following antibodies: anti-CD3 PerCP (clone: BW 264/56, MiltenyiBiotec), anti-CD56 phosphatidylethanolamine (PE)-Cy7 (clone: B159, BD Pharmingen), anti-CD57 VioBlue (clone: TB03, MiltenyiBiotec), anti-CD300a PE (clone: E59.126, Beckman Coulter), anti-CD4 FITC

TABLE 1 | Demographics of studied individuals ($n = 64$).

CMV	Age (years)	No.	Group name
Negative	18–35	22	Young CMV-seronegative
Positive	18–35	15	Young CMV-seropositive
Positive	40–65	13	Middle age CMV-serpositive
Positive	>70	14	Old CMV-serpositive

(clone: M-T466, MiltenyiBiotec), anti-CD8 APC-Cy7 (clone: SK1, BD Biosciences), and anti-CD161 APC (clone: DX12, BD Pharmingen). All antibodies were titrated before use.

Samples were acquired with a nine parameters MACsQuant instrument (Miltenyi Biotech, BergischGladbach, Germany) and analyzed with FlowJo v X 10.0.7 software (TreeStar, Portland, OR, USA). First, lymphocytes were gated according to their size and granularity (FSC vs SSC), then forward scatter height versus forward scatter area to remove doublets. Within that gate (singlets), $CD3^+$ T-cells were gated, followed by identification of the different T-cell subsets by confronting CD4 vs CD8. NKT-like cells ($CD8^+CD56^+$) were then gated from $CD8^+$ T-cells (Figure S1A in Supplementary Material). The average number of events acquired for each subset was: 71161 cells for $CD4^+$ subset, 32498 cells for $CD8^+$, 5708 cells for NKT-like, and 5511 cells for DN. Individual gates (set based on fluorescence minus one controls) for $CD57^+$, $CD161^+$, and $CD300^+$ cells were gated on each of these populations (Figure S1B in Supplementary Material). FlowJo's Boolean gating options were performed to analyze the co-expression of CD57, CD161, and CD300a markers.

Statistical Analysis

Data were inspected for normal distribution using the Shapiro–Wilk test. No normality was found. According to this,

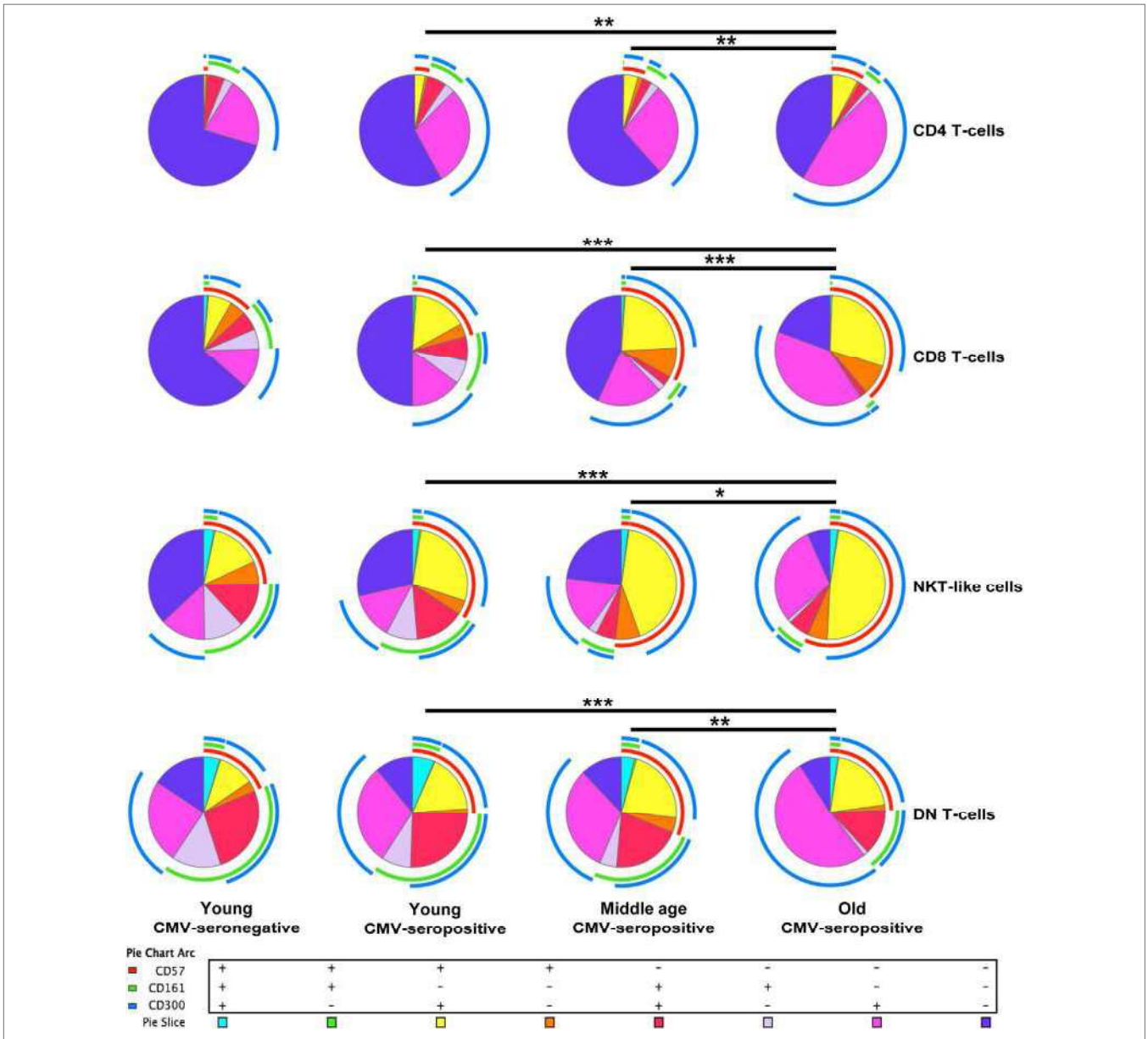

FIGURE 1 | Expression of CD57, CD161, and CD300a in T-cells. CD57, CD161, and CD300a co-expression patterns (pie charts) in $CD4^+$, $CD8^+$, NKT-like, and DN T-cells from healthy individuals ($n = 64$), stratified by age and CMV serostatus. Results were considered significant at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

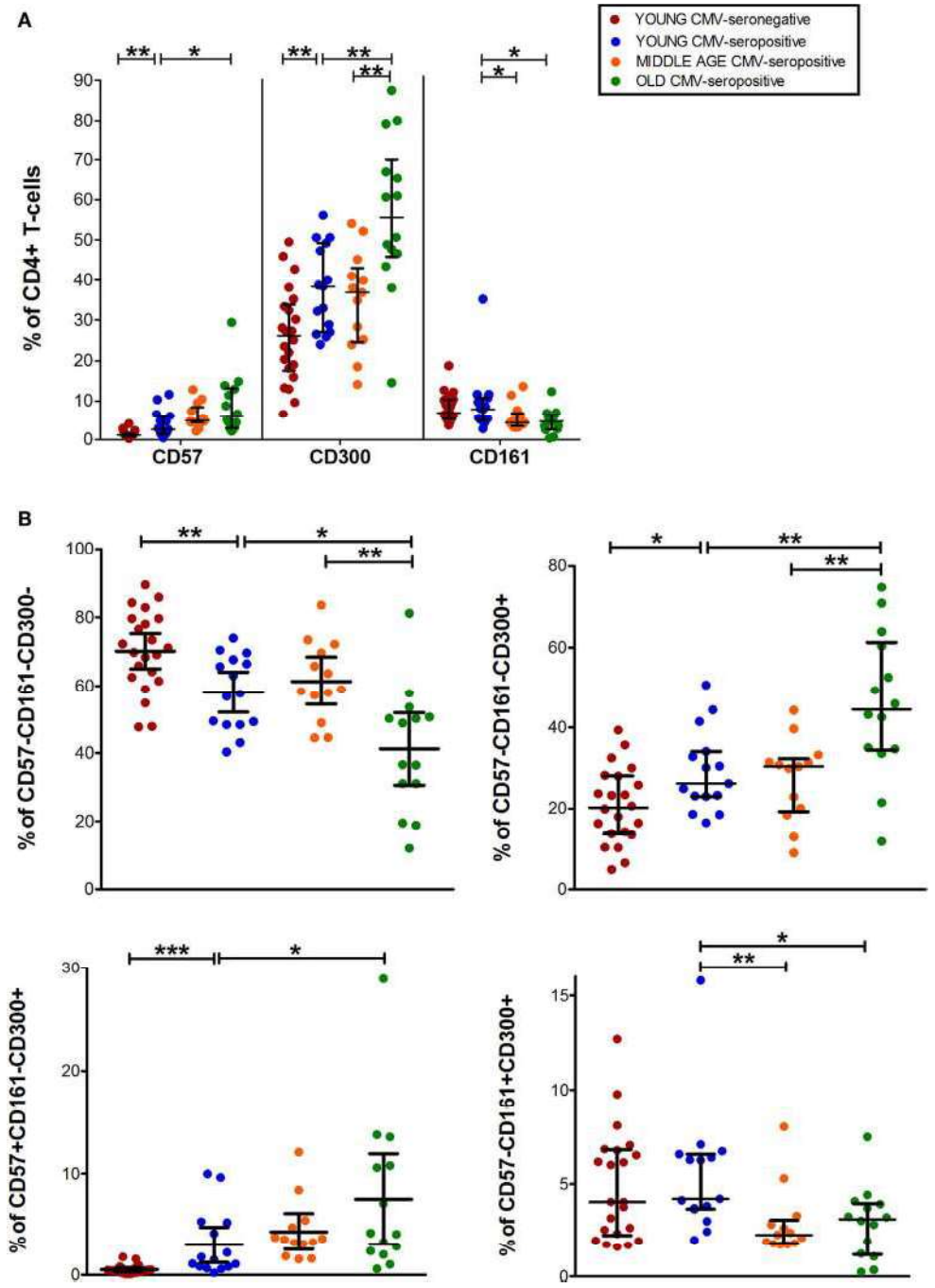

FIGURE 2 | CD57, CD300, and CD161 expression on CD4+ T-cells. **(A)** Total expression (percentage) of CD57, CD161, and CD300a on CD4+ T-cells from young CMV-seronegative ($n = 22$), young CMV-seropositive ($n = 15$), middle age CMV-seropositive ($n = 13$), and old CMV-seropositive donors ($n = 14$). **(B)** Co-expression of CD57, CD161, and CD300a on CD4+ T-cells. Graphs show CD4+ T-cell phenotypes in which we found statistical differences among the four groups studied. Vertical blacklines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line. Results were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Kruskal–Wallis *H* test (non-parametric test) with correction for multiple comparisons was used for direct comparison of the four groups. Those variables in which we found a statistical significant difference were then analyzed using the Mann–Whitney *U* non-parametric test for comparing data among the specific sample pairs. All statistical tests were performed with PASW Statistics v18. For scatter graphs, GraphPad Prism (version 5.0) was used. All graphs reflect only the Mann–Whitney derived *p*-values. To compare the pie charts, we used SPICE's permutation analysis (Mario Roederer, ImmunoTechnology Section, Vaccine

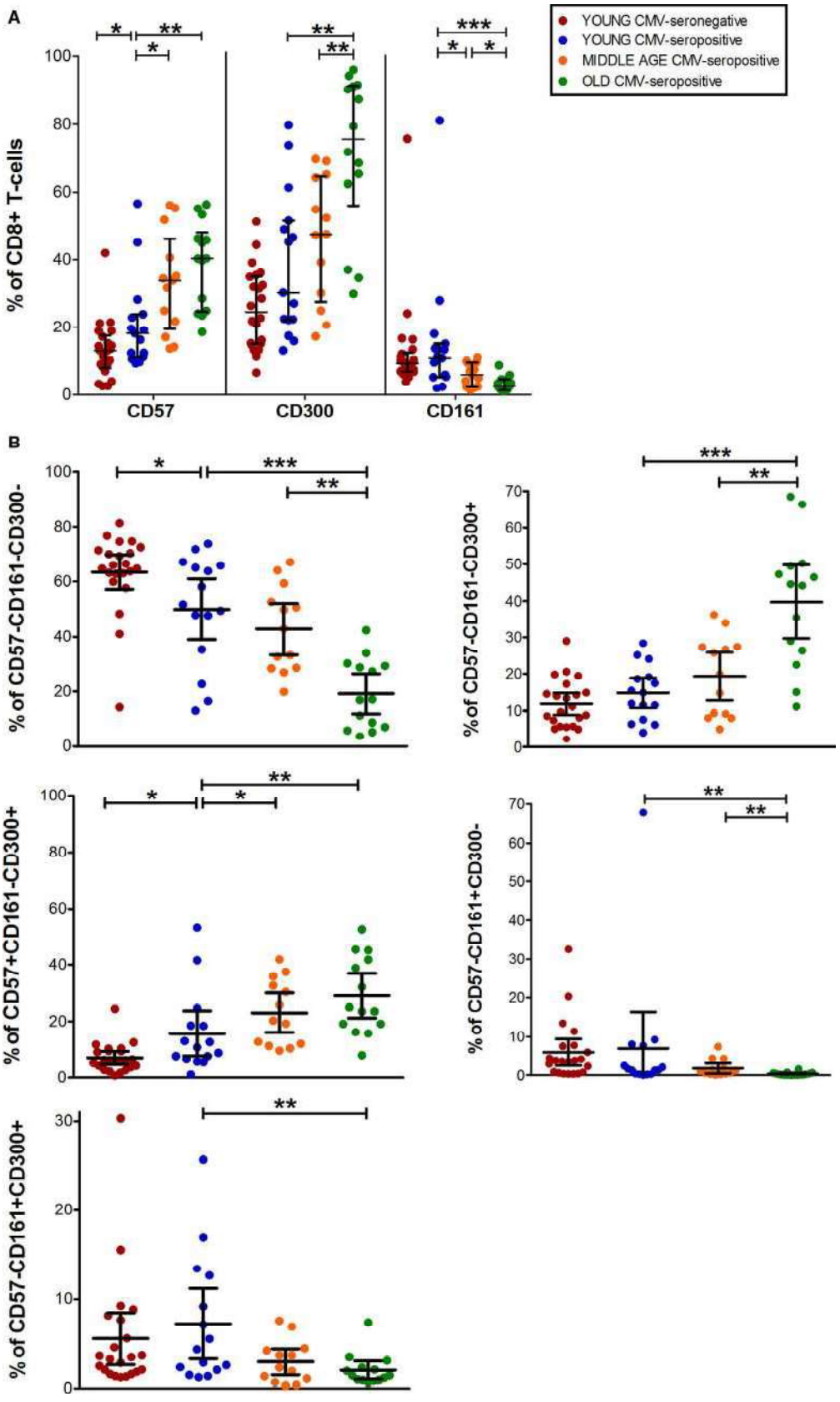

FIGURE 3 | Continued

FIGURE 3 | Continued
CD57, CD300, and CD161 expression on CD8+ T-cells. **(A)** Total expression (percentage) of CD57, CD161, and CD300a markers on CD8+ T-cells from young CMV-seronegative ($n = 22$), young CMV-seropositive ($n = 15$), middle age CMV-seropositive ($n = 13$), and old CMV-seropositive donors ($n = 14$). **(B)** Co-expression of CD57, CD161, and CD300a on CD8+ T-cells. Graphs show the phenotype combinations CD8+ T-cells in which we found statistical differences among the four groups studied. Vertical blacklines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line. Results were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Research Centre, NIH, Bethesda, MD, USA) (48), which asks how often given the samples that comprise the two pies charts, the difference observed would happen simply by chance (10,000 permutations).

RESULTS

CD57, CD161, and CD300a Expression on T-Cells

Multicolor flow cytometry was used to analyze the expression of CD57, CD161, and CD300a markers on CD4+, CD8+, NKT-like, and DN T-cell subpopulations from healthy individuals stratified by age and CMV-serostatus (**Table 1**).

FlowJo's Boolean analysis of CD57, CD161, and CD300a expression generated eight different possible phenotype combinations per T-cell subset. However, not all the possible combinations were biologically meaningful. Phenotype profiles for each subset were obtained using SPICE software (**Figure 1**).

CD4+ T-Cells

Analysis of CD4+ T-cell subset showed that CD57+CD4+ and CD300a+CD4+ T-cells increased with age in CMV-seropositive individuals and with CMV infection in young individuals (**Figure 2A**). In contrast, CD161+CD4+ T-cells percentage was decreased with age (**Figure 2A**).

Out of the eight possible Boolean phenotype combinations, we only found five within the CD4+ T-cell subset, as the percentages of cells with CD57+CD161+CD300a+, CD57+CD161+CD300a− and CD57+CD161−CD300a− phenotypes were noticeably low or null in all subjects studied (**Figure 1**).

The majority of CD4+ T-cells in young and middle-age individuals did not express any of the markers studied (CD57−CD161−CD300a−). However, in the elderly, more than 50% of the cells were CD300a+ (55.67%, IQR 46.62–66.99) alone or in combination with CD161 or CD57 (**Figures 1** and **2A**, Table S1 in Supplementary Material). Our data as well showed that the percentage of triple negative (CD57−CD161−CD300a−) CD4+ T-cells was decreased by CMV infection in young individuals. The progressive reduction of CD57−CD161−CD300a− CD4+ T-cells by CMV infection and age corresponded with an increase of CD57−CD161−CD300a+ and CD57+CD161−CD300+ phenotypes (**Figure 2B**). Of note, CD57+CD4+ T-cells were only present in CMV-seropositive individuals and always co-expressing CD300a (**Figures 1** and **2B**).

On the other hand, our analysis showed that CD161 is never co-expressed with CD57 in any of the groups studied (**Figure 1**). CD57−CD161+CD300a− and CD57−CD161+CD300a+ CD4+ T-cells decreased with age in CMV-seropositive individuals, being the percentage of CD57−CD161+CD300a− cells very low or null in the elderly (**Figures 1** and **2B**).

Furthermore, the phenotype profiles of CD4+ T-cells changed with age in CMV-seropositive individuals, but not with CMV infection alone (pie charts representing the three makers' combinations, **Figure 1**). This shift of phenotype is mainly due to an accumulation with age of the CD57−CD161−CD300a+ and CD57+CD161−CD300a+ phenotypes in the CMV-seropositive individuals.

CD8+ T-Cells

Data from CD8+ T-cell subset showed that CD57+CD8+ T-cells increased with CMV infection alone and in combination with age. While, CD300a+CD8+ T-cells accumulate with age in CMV-seropositive individuals and CD161+CD8+ T-cells decreased progressively being very low or null in the elderly (**Figures 1** and **3A**).

The percentages of CD57+CD161+CD300a+ and CD57+CD161+CD300a− CD8+ T-cells were noticeably low or null in all subjects studied. The majority of CD57+CD8+ T-cells were positive for CD300a and negative for CD161 (**Figure 1**). However, in contrast to CD4+ T-cells, in the CD8+ subset, we found a small fraction of cells with CD57+CD161−CD300a− phenotype (pie slice orange, **Figure 1**), not affected by age.

In young and middle age individuals, 60–70% of the CD8+ T-cells were mainly CD57−CD161−CD300a− (Table S1 in Supplementary Material). However, in the elderly, only 17% (IQR 7.20–29.30) of CD8+ T-cells did not express any of the markers (**Figure 3B**; Table S1 in Supplementary Material). This drastic reduction observed in the elderly is due to the expansion of CD300a+ cells with or without CD57 (yellow and pink pie slices, **Figure 1**). In young individuals, CD57−CD161−CD300a− CD8+ T-cells decreased with CMV infection (**Figure 3B**) due to the expansion of CD57+CD161−CD300a+ cells (yellow pie slice, **Figures 1** and **3B**).

Additionally, we observed that in young and middle age individuals, CD161+CD8+ T-cells were CD300a+ or CD300a−, whereas in the elderly, the few CD161+ cells observed were all CD300a+ (pie slices red and violet, **Figure 1**).

The phenotype profiles (pie charts, **Figure 1**) of CD8+ T-cells changed noticeably with age in CMV-seropositive individuals, but not with CMV infection alone (**Figure 1**).

CD8+CD56+ T-Cells (NKT-Like Cells)

The expression of CD57, CD161, and CD300a markers on NKT-like cells was not affected by CMV infection alone. However, CD57+ and CD300a+ NKT-like cells increased with age in CMV-seropositive individuals (**Figure 4A**), while CD161+ NKT-like cells decreased (**Figure 4A**).

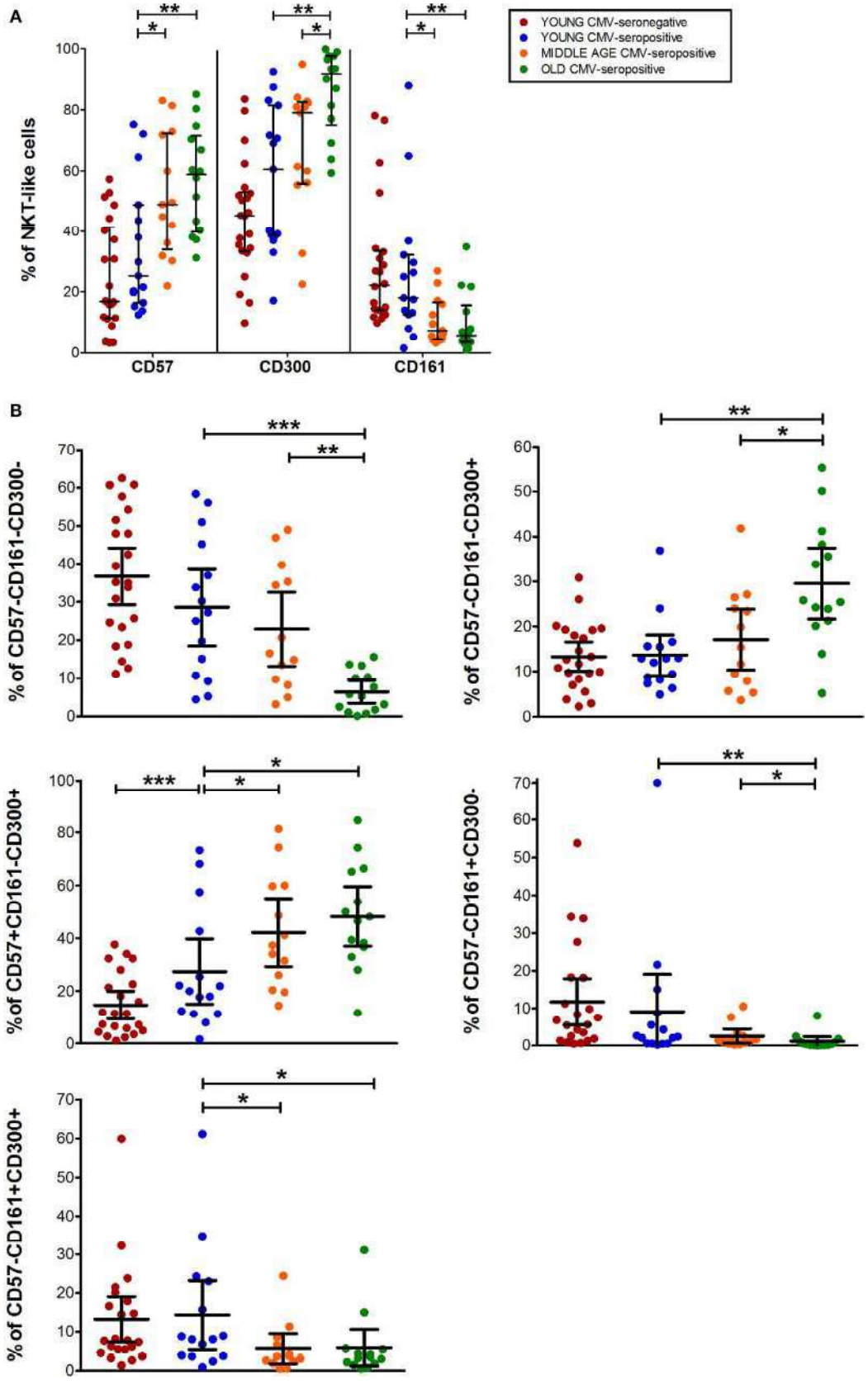

FIGURE 4 | Continued

FIGURE 4 | Continued
CD57, CD300, and CD161 expression on NTK-like cells. **(A)** Total expression (percentage) of CD57, CD161, and CD300a markers on NKT-like cells from young CMV-seronegative ($n = 22$), young CMV-seropositive ($n = 15$), middle age CMV-seropositive ($n = 13$), and old CMV-seropositive donors ($n = 14$). **(B)** Co-expression of CD57, CD161, and CD300a on NKT-Like cells. Graphs show the markers combinations in which we found statistical differences among the four groups studied. Vertical black lines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line. Results were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

$CD57^+CD161^+CD300a^+$ and $CD57^+CD161^+CD300a^-$ NKT-like cells were very low or null. Thus, in our hands, as $CD4^+$ and $CD8^+$ subsets, NKT-like cells did not co-express CD57 and CD161 (pie slice orange, **Figure 1**). The majority of $CD57^+$ NKT-like cells were also $CD300a^+$. However, we observed a small fraction of NKT-like cells with a $CD57^+CD161^-CD300a^-$ phenotype, not affected by CMV infection and age (**Figure 1**). Of note, in the elderly, 47.35% (IQR 36.70–65.10) of the NKT-like cells were $CD57^+CD161^-CD300a^+$ (yellow pie slice, **Figure 1**). The proportion of this phenotype is significantly lower ($p < 0.001$) in the rest of the T-cell subsets studied, particularly in the $CD4^+$ T-cells in which this phenotype frequency is quite low even in the elderly (**Figure 1**).

In the NKT-like subset, 65–95% of the cells expressed at least one of the markers studied, being the fraction of triple negative cells ($CD57^-CD161^-CD300a^-$) very low in old individuals (5.58%, IQR 1.77–10.20) (**Figures 1** and **4B**), due to the expansion of $CD57^-CD161^-CD300a^+$ and $CD57^+CD161^-CD300a^+$ phenotypes (pie slices pink and yellow, **Figure 1**). Noticeably, $CD57^+CD161^-CD300a^+$ cells were also increased in young CMV-seropositive individuals compared with CMV-seronegative (**Figure 4B**).

NKT-like $CD161^+$ phenotypes ($CD57^-CD161^+CD300a^-$ and $CD57^-CD161^+CD300a^+$) decreased with age in CMV-seropositive individuals, but not with CMV infection alone (**Figures 1** and **4B**). Remarkably, the percentage of $CD57^-CD161^+CD300a^-$ NKT-like cells was very low or null in middle age and old individuals (**Figure 4B**; Table S1 in Supplementary Material).

As in the $CD4^+$ and $CD8^+$ main populations, the phenotype profiles of NKT-like cells were not affected by CMV infection alone (**Figure 1**).

$CD4^-CD8^-$ T-Cells (DN T-Cells)

Data from DN T-cells (majorly γδ T-cells) flow analysis showed a similar percentage of $CD57^+$ DN T-cells among the three CMV-seropositive groups (young, middle age, and old) (**Figure 5A**). While, $CD161^+$ DN T-cell decreased gradually with age in CMV-seropositive individuals (**Figure 5A**). Furthermore, $CD300a^+$ DN T-cells increased with CMV infection in young individuals and further increased in old CMV-seropositive individuals (**Figure 5A**).

As in the other T-cell subsets studied, in DN T-cells the percentage of $CD57^+CD161^+CD300a^-$ cells is null (**Figure 1**). However, we observed a small fraction of DN T-cells co-expressing the three markers that decreases with age (**Figures 1** and **5B**).

The majority of DN T-cells in all individuals are $CD300a^+$ with or without CD161 or CD57 expression (**Figure 1**). Our analysis showed that the percentage of $CD57^-CD161^-CD300a^+$ cells increased with age in CMV-seropositive individuals, whereas the percentage of $CD57^+CD161^-CD300a^+$ increases with CMV infection in young individuals and is not affected by age (**Figure 5B**). Of note, our results showed that, similarly to $CD57^+CD4^+$ T-cells, the majority of $CD57^+$ DN T-cells are as well $CD300^+$ (**Figure 1**).

Besides, $CD161^+$ DN T-cells were mainly $CD57^-$ (only a small fraction co-expressed CD161 and CD57). $CD57^-CD161^+CD300a^-$ phenotype decreased with age, being the percentage of these cells null in the elderly (pie slice violet, **Figures 1** and **5B**). Whereas a reduced percentage of $CD57^-CD161^+CD300a^+$ DN T-cells was still present in old individuals (pie slice red, **Figures 1** and **5B**).

DN T-cell phenotype profiles for the makers studied (pie charts, **Figure 1**) changed with age in CMV-seropositive individuals, but not with CMV infection alone.

DISCUSSION

The combination of age and CMV latent infection has been proven to have a profound impact on the immune phenotype and function of T-cells, not only on the $CD8^+$ subset but also on $CD4^+$, NKT-like, and γδ T-cells. However, age and CMV infection do not always have similar effects and it can vary depending on the cell type.

Here, we analyzed, in different T-cell subsets, how age and CMV infection alter the expression of the inhibitory receptors CD300a and CD161 and their relation with the marker CD57, which has been shown to be a polyfunctionality maker of $CD4^+$, $CD8^+$, and NKT-like T-cells (37, 38, 49). We are aware that due to the high prevalence of CMV in our geographic area (see Materials and Methods), a limitation of our study is the lack of CMV-seronegative individuals of older ages (middle age and old groups). Thus, we can only assess the effect of aging in the context of CMV latent infection. Nevertheless, we were able to investigate the effect of CMV infection alone in young individuals.

Our analysis showed that in all T-cell subsets studied, CD57 and CD300a increase with age in CMV-seropositive individuals. Specifically, with CMV infection (young individuals), CD57 is increased only in $CD4^+$ and $CD8^+$ T-cells and CD300a in $CD4^+$ and DN subsets. No effect of CMV alone was observed on NKT-like cells. Of note, $CD57^+CD4^+$ T-cells are always $CD300a^+$ and were only found in CMV-seropositive individuals. In the rest of subsets ($CD8^+$, NKT-like and DN), although not all, the majority of $CD57^+$ T-cells were $CD300a^+$ as well, regardless of the age and CMV serostatus.

The expression of CD300a by several immune cell types has been associated with different pathologies, suggesting that, although the significance of CD300a on T-cell function is not completely clear, CD300a could be used as a biomarker and a target for new therapies [for review, see Ref. (50)].

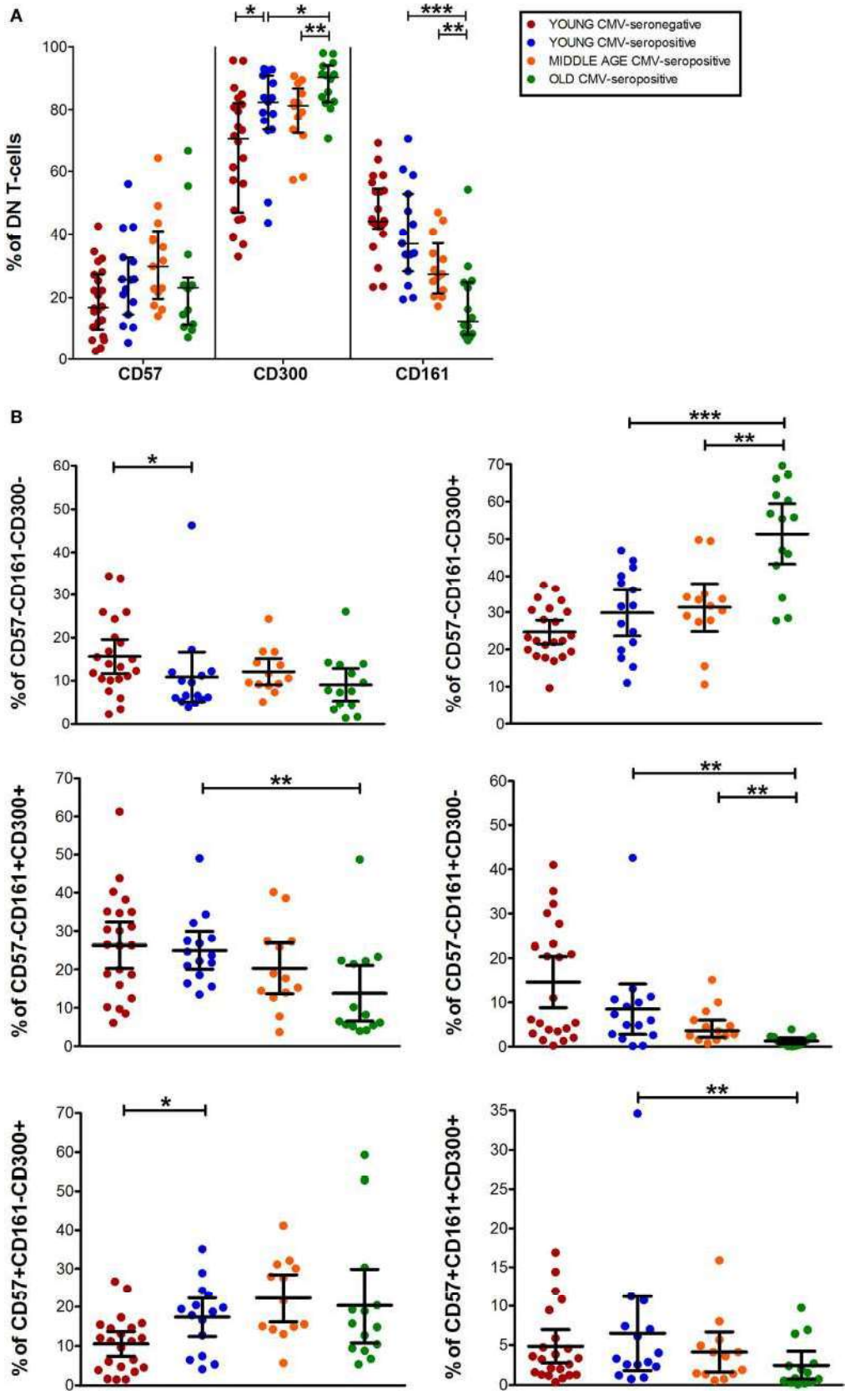

FIGURE 5 | Continued

FIGURE 5 | Continued
CD57, CD300, and CD161 expression on $CD4^-CD8^-$ T-cells (DN). **(A)** Total expression (percentage) of CD57, CD161, and CD300a markers on DN T-cells from young CMV-seronegative ($n = 22$), young CMV-seropositive ($n = 15$), middle age CMV-seropositive ($n = 13$), and old CMV-seropositive donors ($n = 14$). **(B)** Coexpression of CD57, CD161, and CD300a on DN T-cells. Graphs show DN T-cells phenotypes in which we found statistical differences among the four groups studied. Vertical black lines indicate interquartile ranges, ranging from the 25th to the 75th percentile. The median values are indicated by a horizontal black line. Results were considered significant at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

CD300a ligands, phosphatidylserine (PS), and phosphatidylethanolamine (PE) are associated with virus evasion. Indeed, anti-PS antibody has been shown to be a potential treatment for CMV and Pichinde virus infections (51). Additionally, HIV-specific $CD8^+$ T-cell mRNA levels of CD300a receptor have been shown to correlate with the expression of BATF transcription factor, which is highly expressed in exhausted cells. BTAF inhibits cell function by inducing the expression of inhibitory receptors such as CD300a (52). Viral envelope expression of PS and PE has been shown to be an evasion mechanism called "apoptotic mimicry" (53). However, CD300a binding to viruses expressing PS and PE in their envelopes seams to inhibit the virus endocytosis, most probably hampering the virus infection. All these data supports that CD300a has an inhibitory role and it is important for viral infections.

However, in $CD8^+$ T-cells, CD300a expression associates with higher cytotoxicity and $CD300a^+CD8^+$ T-cells are increased in pregnant women with chronic choriamnionitis (10). Additionally, CD300a has been shown to be a polyfunctionality marker in $CD4^+$ T-cells and $CD300a^+CD4^+$ T-cells upregulate Eomes transcription factor after stimulation (11). Furthermore, our group has recently shown that $CD57^+CD4^+$ T-cells are polyfunctional and express high levels of T-bet and Eomes transcription factors upon superantigen stimulation (38). Moreover, $CD57^+CD8^+$ T-cells correlate with polyfunctionality of $CD8^+$ T-cells and are expanded in young CMV-seropositive individuals (37).

Whether $CD300a^+CD57^-$ and $CD300a^+CD57^+$ T-cells display any differences regarding polyfunctionality and if there are differences in regards of T-bet and Eomes expression is currently under investigation in our laboratory. This analysis will allow us to establish if CD300a is a polyfunctional marker of T-cells *per se*, or only if co-expressed with CD57. In our hands $CD57^+$ T-cells co-expressing CD300a expand with CMV infection (in young individuals), highlighting a relevant role for both makers in the control of CMV virus by T-cells. On the other hand, CD161 receptor was hardly co-expressed with CD57 in any of the T-cell subsets studied. Particularly, co-expression of CD161 and CD57 was not observed in the elderly regardless of the T-cell subset. Furthermore, the total expression of CD161, contrarily to CD57 and CD300a, decreases with age in CMV-seropositive individuals and is not affected by CMV infection alone in young individuals. Our results support previous results from healthy children in which the expression of CD161 on T and NK cells was not affected by CMV serostatus (54). However, Almehmadi et al. suggest that NKT-like cells not expressing CD161 are increased in CMV-seropositive individuals. This discrepancy with our data can be explained by the fact that Almehmadi's cohort does not stratify the individuals by age (23–60 years), only by CMV status. Additionally, their definition of NKT-like cells differs from ours as it is based only on CD3 and CD56 expression, not including CD8 [for review of NKT-like cells nomenclatures, see Ref. (55)]. In our previous work, regarding NKT-like cell number and functionality in the context of CMV infection and age, we show that the percentage of NKT-like cells is not affected by CMV infection in young CMV individuals, but rather with the combined effect of both age and CMV latent infection (49). Similarly, the loss of CD161 by T-cells does not associate with CMV alone, but with age in the context of CMV latent infection. Indeed, the expression of CD161 in CMV-specific cytotoxic T lymphocytes is very low (56).

Besides, acute and chronic GVHD correlates with decreased levels of circulating $CD161^+CD4^+$ and $CD161^{++}CD8^+$ T-cells (57). Moreover, in rheumatoid arthritis patients, it has been shown an increase of $CD161^+CD4^+$ T-cells, but a decrease of $CD161^+$ DN T-cells that was associated with disease activity and inflammation (58, 59). As we mentioned before, T-cells-expressing CD161 are IL-17 producers. In our cohort, the percentages of $CD161^+$ T-cells are very low or null in old CMV-seropositive individuals regardless of the T-cell type. This could translate in a diminished Th17 response in the elderly.

Contrarily to what we observe in T-cells, our previous work on NK cells showed a decreased expression of CD161 on CD56dim NK cells associated with CMV seropositivity (60).

The data presented here together with our previous results highlight the importance of taking into account both age and CMV serostatus in any clinical study regarding the analysis of T-cells, as CMV latent infection has a differential effect with age on T-cell subsets. Additionally, our data support the potential use of CD57, CD300a, and CD161 as biomarkers of immunosenescence and as possible targets for novel therapies. The clinical implications of the changes found in the expression of these makers should be further investigated.

ETHICS STATEMENT

This work was approved by the Ethics Committee of the Reina Sofia University Hospital. All participants in the study provided informed written consent.

AUTHOR CONTRIBUTIONS

RS and AP designed the study. FH collected the data and performed the laboratory experiments. FH and NL-S collaborated in the laboratory analysis. AP and FH performed the statistical analysis and wrote the draft. RT, BS-C, and CC made significant technical and conceptual contributions to the manuscript. RS, RT, and AP reviewed the final version of the paper. All the authors provided intellectual content and approved the final version of

the paper. RS an AP are co-senior authors and have contributed equally to this work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00649/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

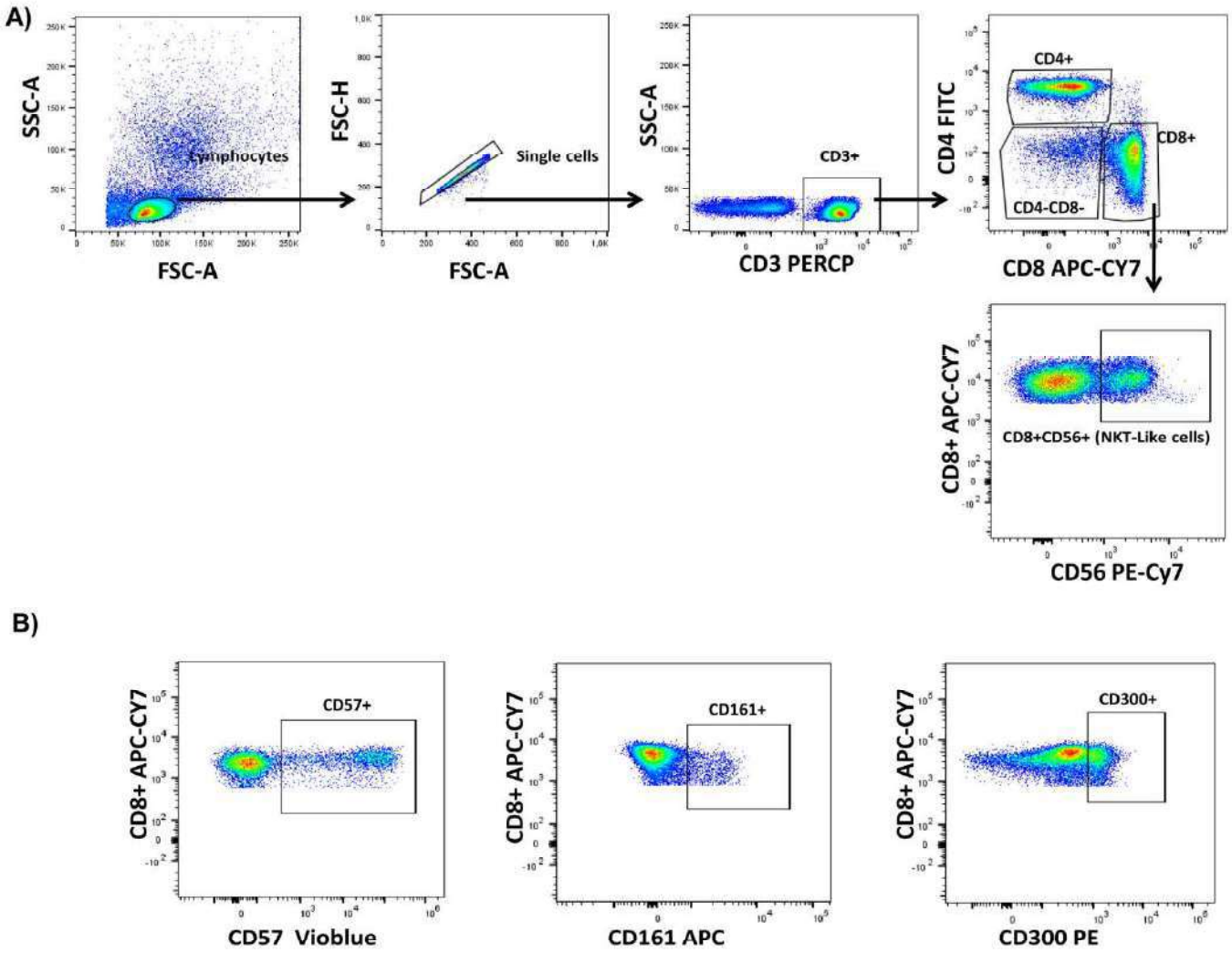

Figure S1. Gating strategy used for the analysis of CD57, CD161 and CD300a expression on T-cells. A) Peripheral blood lymphocytes (PBLs) were selected using forward (FSC) and side scatter (SSC) detectors, subsequently CD3+ T-cells were gated from PBLs after single cells gating , followed by identification of CD4+, CD8+, CD4–CD8– T-cells (DN: Double negative) and CD8+CD56+ T-cells (NKT-Like cells). B) Total expression of CD57, CD161 and CD300a was determined within each T-cell subset using Fluorescence minus one (FMO) controls. Figure shows an example for CD8+ T-cell subset.

Table S1. Median and InterQuartile Range (IQR) of the Co-expression analysis of CD57, CD300a and CD161 on T-cell subsets A) CD4 and CD8 phenotypes

CMV status	CD4+ phenotype	Median	Q1 Percentile 25	Q3 Percentile 75	CD8+ phenotype	Median	Q1 Percentile 25	Q3 Percentile 75
Young CMV-seronegative	CD4+CD57+CD161+CD300+	.07	.04	.11	CD8+CD57+CD161+CD300+	.49	.21	.72
	CD4+CD57+CD161+CD300-	.03	.02	.04	CD8+CD57+CD161+CD300-	.16	.06	.28
	CD4+CD57+CD161-CD300+	.41	.22	.60	CD8+CD57+CD161-CD300+	5.94	3.66	9.37
	CD4+CD57+CD161-CD300-	.49	.40	.69	CD8+CD57+CD161-CD300-	2.98	1.28	7.60
	CD4+CD57-CD161+CD300+	4.05	2.35	6.80	CD8+CD57-CD161+CD300+	3.34	1.81	7.64
	CD4+CD57-CD161+CD300-	2.73	2.33	3.62	CD8+CD57-CD161+CD300-	3.69	.76	7.50
	CD4+CD57-CD161-CD300+	20.25	13.80	28.00	CD8+CD57-CD161-CD300+	10.42	5.79	14.80
	CD4+CD57-CD161-CD300-	70.50	62.60	79.70	CD8+CD57-CD161-CD300-	65.30	62.80	71.40
Young CMV-seropositive	CD4+CD57+CD161+CD300+	.10	.05	.33	CD8+CD57+CD161+CD300+	.70	.35	.96
	CD4+CD57+CD161+CD300-	.02	.01	.06	CD8+CD57+CD161+CD300-	.08	.03	.21
	CD4+CD57+CD161-CD300+	1.51	.83	5.23	CD8+CD57+CD161-CD300+	10.70	6.48	18.50
	CD4+CD57+CD161-CD300-	.55	.36	.64	CD8+CD57+CD161-CD300-	3.25	1.48	5.58
	CD4+CD57-CD161+CD300+	4.22	3.66	6.59	CD8+CD57-CD161+CD300+	4.33	2.05	12.60
	CD4+CD57-CD161+CD300-	2.11	1.05	4.05	CD8+CD57-CD161+CD300-	1.40	.29	7.68
	CD4+CD57-CD161-CD300+	26.20	23.00	34.10	CD8+CD57-CD161-CD300+	14.80	7.61	19.10
	CD4+CD57-CD161-CD300-	57.60	48.30	67.70	CD8+CD57-CD161-CD300-	51.80	35.30	65.80
Middle age CMV-seropositive	CD4+CD57+CD161+CD300+	.23	.07	.33	CD8+CD57+CD161+CD300+	.60	.35	.96
	CD4+CD57+CD161+CD300-	.02	.02	.04	CD8+CD57+CD161+CD300-	.16	.04	.42
	CD4+CD57+CD161-CD300+	3.57	3.12	4.77	CD8+CD57+CD161-CD300+	20.30	12.00	32.50
	CD4+CD57+CD161-CD300-	.70	.63	1.11	CD8+CD57+CD161-CD300-	7.34	2.17	11.60
	CD4+CD57-CD161+CD300+	2.29	1.82	2.83	CD8+CD57-CD161+CD300+	2.28	1.08	4.19
	CD4+CD57-CD161+CD300-	1.72	1.22	2.30	CD8+CD57-CD161+CD300-	1.10	.39	1.79
	CD4+CD57-CD161-CD300+	30.40	20.10	31.40	CD8+CD57-CD161-CD300+	19.90	9.14	27.30
	CD4+CD57-CD161-CD300-	58.90	57.10	69.70	CD8+CD57-CD161-CD300-	42.90	28.60	52.70
Old CMV-seropositve	CD4+CD57+CD161+CD300+	.13	.08	.40	CD8+CD57+CD161+CD300+	.39	.30	.82
	CD4+CD57+CD161+CD300-	.02	.01	.04	CD8+CD57+CD161+CD300-	.03	.00	.09
	CD4+CD57+CD161-CD300+	4.15	2.36	10.80	CD8+CD57+CD161-CD300+	24.25	19.10	41.80
	CD4+CD57+CD161-CD300-	.71	.42	1.33	CD8+CD57+CD161-CD300-	2.68	1.14	11.10
	CD4+CD57-CD161+CD300+	3.14	1.23	3.93	CD8+CD57-CD161+CD300+	1.42	.91	2.28
	CD4+CD57-CD161+CD300-	1.15	.55	2.52	CD8+CD57-CD161+CD300-	.22	.07	.61
	CD4+CD57-CD161-CD300+	44.55	34.70	60.40	CD8+CD57-CD161-CD300+	44.30	26.40	49.40

Table S1. Median and InterQuartile Range (IQR) of the Co-expression analysis of CD57, CD300a and CD161 on T-cells subsets B) NKT-like and DN phenotypes

CMV status	NKT-like phenotype	Median	Q1 Percentile 25	Q3 Percentile 75	DN phenotype	Median	Q1 Percentile 25	Q3 Percentile 75
Young CMV-seronegative	CD8+CD56+CD57+CD161+CD	1.20	.56	3.79	CD4-CD8-	3.29	1.29	5.88
	CD8+CD56+CD57+CD161+CD	.26	.06	.82	CD4-CD8-	.19	.05	.52
	CD8+CD56+CD57+CD161-	11.40	4.91	22.60	CD4-CD8-CD57+CD161-	11.20	4.53	14.60
	CD8+CD56+CD57+CD161-	2.44	1.15	10.20	CD4-CD8-CD57+CD161-	2.15	1.00	3.77
	CD8+CD56+CD57-	7.72	5.42	17.90	CD4-CD8-CD57-	26.40	16.00	35.00
	CD8+CD56+CD57-	6.15	1.83	18.10	CD4-CD8-CD57-	8.56	3.48	23.20
	CD8+CD56+CD57-CD161-	12.20	8.37	19.20	CD4-CD8-CD57-CD161-	22.75	19.40	30.60
	CD8+CD56+CD57-CD161-	35.10	23.40	51.60	CD4-CD8-CD57-CD161-CD300-	13.55	10.40	20.00
Young CMV-seropositive	CD8+CD56+CD57+CD161+CD	1.34	1.00	2.50	CD4-CD8-	3.31	2.29	7.41
	CD8+CD56+CD57+CD161+CD	.17	.02	.50	CD4-CD8-	.07	.03	.40
	CD8+CD56+CD57+CD161-	20.00	11.20	42.70	CD4-CD8-CD57+CD161-	18.70	7.43	23.10
	CD8+CD56+CD57+CD161-	3.03	.94	6.19	CD4-CD8-CD57+CD161-	1.66	.68	2.19
	CD8+CD56+CD57-	8.09	3.77	23.00	CD4-CD8-CD57-	23.70	18.60	28.10
	CD8+CD56+CD57-	2.32	.50	8.69	CD4-CD8-CD57-	5.94	2.60	10.70
	CD8+CD56+CD57-CD161-	12.90	8.27	15.60	CD4-CD8-CD57-CD161-	31.60	19.80	40.00
	CD8+CD56+CD57-CD161-	27.20	10.70	45.20	CD4-CD8-CD57-CD161-CD300-	6.59	5.65	12.00
Middle age CMV-seropositive	CD8+CD56+CD57+CD161+CD	1.24	.93	1.65	CD4-CD8-	3.62	1.40	4.93
	CD8+CD56+CD57+CD161+CD	.19	.05	.29	CD4-CD8-	.21	.02	.55
	CD8+CD56+CD57+CD161-	37.30	26.00	59.70	CD4-CD8-CD57+CD161-	21.60	14.90	30.10
	CD8+CD56+CD57+CD161-	5.16	2.12	10.20	CD4-CD8-CD57+CD161-	3.02	1.70	4.89
	CD8+CD56+CD57-	3.67	2.48	6.65	CD4-CD8-CD57-	17.70	14.00	27.40
	CD8+CD56+CD57-	1.40	.71	2.54	CD4-CD8-CD57-	3.52	2.42	5.99
	CD8+CD56+CD57-CD161-	15.40	7.95	24.00	CD4-CD8-CD57-CD161-	31.70	27.80	34.10
	CD8+CD56+CD57-CD161-	16.50	9.75	35.30	CD4-CD8-CD57-CD161-CD300-	10.60	9.16	14.20
old CMV-seropositve	CD8+CD56+CD57+CD161+CD	1.15	.87	2.75	CD4-CD8-	1.19	.37	2.70
	CD8+CD56+CD57+CD161+CD	.01	.00	.10	CD4-CD8-	.00	.00	.02
	CD8+CD56+CD57+CD161-	47.35	36.70	65.10	CD4-CD8-CD57+CD161-	15.25	9.42	20.40
	CD8+CD56+CD57+CD161-	2.07	.58	8.92	CD4-CD8-CD57+CD161-	1.06	.70	2.68
	CD8+CD56+CD57-	3.40	1.82	5.47	CD4-CD8-CD57-	7.35	5.46	22.20
	CD8+CD56+CD57-	.34	.10	1.08	CD4-CD8-CD57-	1.05	.33	2.17
	CD8+CD56+CD57-CD161-	25.60	21.30	38.20	CD4-CD8-CD57-CD161-	55.60	42.90	61.80

4.3. Effect of Cytomegalovirus (CMV) and Ageing on T-Bet and Eomes Expression on T-Cell Subsets

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Effect of Cytomegalovirus (CMV) and Ageing on T-Bet and Eomes Expression on T-Cell Subsets

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Abstract: The differential impact of ageing and cytomegalovirus (CMV) latent infection on human T-cell subsets remains to some extent controversial. The purpose of this study was to analyse the expression of the transcription factors T-bet and Eomes and CD57 on CD4+, $CD4^{hi}CD8^{lo}$ and CD8+ T-cell subsets in healthy individuals, stratified by age and CMV serostatus. The percentage of CD4+ T-cells expressing T-bet or Eomes was very low, in particular in CD4+ T-cells from young CMV-seronegative individuals, and were higher in CMV-seropositive older individuals, in both CD57− and CD57+ CD4+ T-cells. The study of the minor peripheral blood double-positive $CD4^{hi}CD8^{lo}$ T-cells showed that the percentage of these T-cells expressing both Eomes and T-bet was higher compared to CD4+ T-cells. The percentage of $CD4^{hi}CD8^{lo}$ T-cells expressing T-bet was also associated with CMV seropositivity and the coexpression of Eomes, T-bet and CD57 on $CD4^{hi}CD8^{lo}$ T-cells was only observed in CMV-seropositive donors, supporting the hypothesis that these cells are mature effector memory cells. The percentage of T-cells expressing Eomes and T-bet was higher in CD8+ T-cells than in CD4+ T-cells. The percentages of CD8+ T-cells expressing Eomes and T-bet increased with age in CMV-seronegative and -seropositive individuals and the percentages of CD57− CD8+ and CD57+ CD8+ T-cells coexpressing both transcription factors were similar in the different groups studied. These results support that CMV chronic infection and/or ageing are associated to the expansion of highly differentiated CD4+, $CD4^{hi}CD8^{lo}$ and CD8+ T-cells that differentially express T-bet and Eomes suggesting that the expression of these transcription factors is essential for the generation and development of an effector-memory and effector T lymphocytes involved in conferring protection against chronic CMV infection.

Keywords: ageing; cytomegalovirus (CMV); CD57; Eomes; T-bet; T-cells

1. Introduction

The ageing of the immune system, referred as immunosenescence, has been observed in both multicellular invertebrate and vertebrate organisms, including humans [1]. As chronological age advances, the immune system becomes less efficient at reacting against pathogens. It has been described that situations involving chronic activation of the immune system, such as viral infections or cancer, can induce changes in the immune system, referred as "early immunosenescence" [2–5]. Among viral infections, persistent cytomegalovirus (CMV) infection has been described to be involved in the process of immunosenescence, contributing to the detriment of the immune response observed

in elderly donors. Thus, it is well known that CMV infection impacts on the T-cell compartment in ageing [6–9].

The transcription factors T-bet (T-box expressed in T-cells) and Eomesodermin (Eomes) belong to the T-box family of transcription factors that play crucial roles in the development of different organs and tissues including the immune system. The studies of T-bet and Eomes in the context of human T-cells are relatively limited. In murine models T-bet was originally involved in promoting Th1 CD4+ T-cell development while inhibiting the Th2 [10] and Th17 lineage commitment [11,12]. T-bet is also known to modulate a number of genes involved in T-cell trafficking and function [13]. Thus, high levels of T-bet have been associated with superior cytotoxic capacity of CD8+ T-cells by upregulation of perforin and granzyme B expression [14,15]. Although T-bet has been involved in sustaining memory T-cells [14], it has been reported that low expression of T-bet shifts the cells into memory-like cells impaired in sensing homeostatic signals from IL-15 [16,17].

The role of T-bet and Eomes in immune cell development and cytolytic function has been previously described. These transcription factors are expressed in different human blood cell subsets, including CD4+ and CD8+ T-cells, γδ T-cells, invariant natural killer (NK) T-cells, NK cells, B cells, and dendritic cells. T-bet expression increases as peripheral cells become more differentiated [18]. Although T-bet and Eomes expression shows some overlap, their functions are not totally reciprocal. In human peripheral blood T-cells, T-bet expression increases as cells differentiate from naive to effector-memory and effector cells. In contrast to CD8+ T-cells, the expression of T-bet and Eomes is very low in CD4+ T-cells. In addition, whereas the percentages of naive cells CD4 and CD8 T-cells expressing T-bet or Eomes is very low, a high percentage of effector-memory and effector CD4 and CD8 T-cells express these factors and the analysis of T-bet and Eomes coexpression shows that in both CD8+ and CD4+ T-cells the majority of effector-memory and effector cells expressing high levels of T bet (T-bet^{hi}) coexpress Eomes+ [18]. The relevance of these transcription factors in the differentiation, development and maintenance of antiviral CD8+ T-cell responses in mice and human is well established [19–21].

In this study, we sought to further characterize the expression of T-bet and Eomes on human CD4 and CD8 T-cell subsets from healthy human donors in the context of ageing and CMV infection. This analysis included the possible relationship with the expression of CD57, originally defined as a marker of senescent T-cells and more recently considered a marker of highly differentiated effector-memory or effector T-cells responsible of cytokine production.

2. Results

2.1. Molecular Signature of T-Cells in the Context of Ageing and CMV Infection

Multicolour flow cytometry was used to analyse the percentage of CD4+, CD4^{hi}CD8^{lo}, and CD8+ T-cells expressing T-bet and Eomes transcription factors, as well as in regard to CD57 expression in those subsets (Figure 1). The analysis was carried out on cells from healthy individuals stratified by age and CMV serostatus (Table 1). FlowJo's Boolean analysis was used to analyse the coexpression of T-bet and Eomes in combination with CD57. However, not all the possible Boolean combinations contained a significant number of cells.

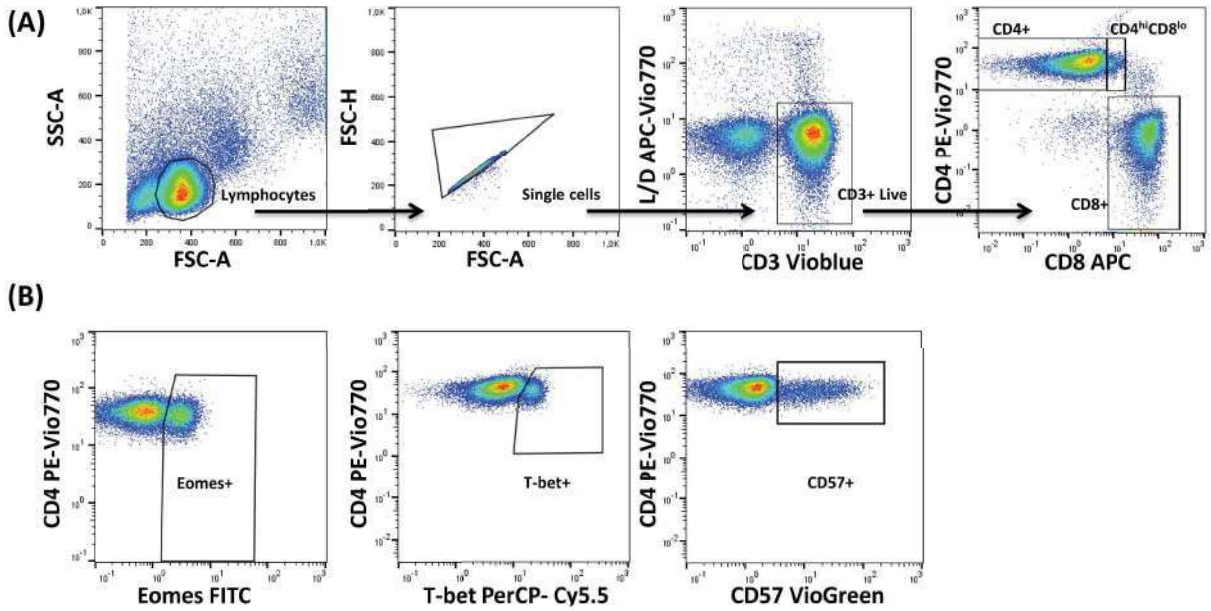

Figure 1. Gating strategy used for the analysis of T-bet, Eomes and CD57 expression on T-cells. (**A**) Peripheral blood lymphocytes (PBLs) were selected using forward (FSC) and side scatter (SSC) detectors. Subsequently Live CD3+ T-cells were gated from PBLs after single cells gating, followed by identification of CD4+, CD8+ and $CD4^{hi}CD8^{lo}$ T-cells. Arrows show the sequence of the gating used, starting from the lymphocytes gate. (**B**) Total expression of Eomes, T-bet and CD57 was determined within each T-cell subset using Fluorescence minus one (FMO) controls. Figure shows a representative example for CD4+ T-cells.

Table 1. Demographics of studied individuals (n = 25).

Group	Age Group	Mean Age (SD)	Sex (Male/Female)
Young CMV-Seronegative	Young (18–35)	31 (6.82)	5/0
Young CMV-Seropositive	Young (18–35)	26 (2.92)	2/3
Middle-aged CMV-Seronegative	Middle-aged (40–60)	54.2 (8.79)	3/2
Middle-aged CMV-Seropositive	Middle-aged (40–60)	52.6 (5.32)	2/3
Elderly CMV-Seropositive	Elderly (>65)	74.6 (10.78)	4/1

2.2. T-Bet and Eomes Expression in CD4+ T-Cells

As some CD4+ T-cells express low levels of CD8 marker and it has been shown to have a mature phenotype distinct from CD4+CD8− T-cells, we decided to analyse both subpopulations separately. Our data showed that although the majority of CD4+CD8− (CD4+) T-cells do not express these transcription factors, in CMV-seropositive and aged individuals CD4+ T-cells expressing both T-bet and Eomes are detectable. Thus, the percentage of cells expressing Eomes increased with CMV infection in young individuals and was higher in the elderly CMV-seropositive (Figure 2, Table 2 and Table S1). The percentage of CD4+T-bet+ T-cells on young CMV-seronegative individuals was null. CD4+ T-cells expressing this transcription factor were higher in CMV-seropositive individuals and older individuals, being particularly high in the older CMV-seropositive individuals.

The Boolean combination of both transcription factors in CD4+ T-cells showed that the percentage of cells expressing Eomes but not T-bet was not significantly affected by age or CMV infection (Figure 3A). Cells expressing only T-bet were less frequent and increased in CMV-seropositive individuals and further increased with age. In a similar way, double-positive Eomes+ T-bet+ cells were very low in young CMV-seronegative individuals and increased in CMV-seropositive donors with maximum values in the elderly CMV-seropositive.

The analysis of T-bet and Eomes according to CD57 expression on CD4+ T-cells (Figure 3B, Table S2) showed that cells expressing both transcription factors and CD57+ are null in young CMV-seronegative. Moreover, although very variable, the highest percentages of these cells were found in the elderly CMV-seropositive. Indeed, CD57+CD4+ T-cells were only found in combination with T-bet with or without Eomes. The majority of CD57−CD4+ T-cells were Eomes+T-bet−. The percentages of these cells remained stable despite CMV infection and age. The percentage of Eomes+CD57−CD4+ T-cells was only affected by age when coexpressing T-bet.

Table 2. Percentage of CD4+ T-cells expressing T-bet and Eomes.

Transcription Factors	Group									
	Young CMV−		Young CMV+		Middle-Aged CMV−		Middle-Aged CMV+		Elderly CMV+	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
EOMES+	3.86	0.70	8.59	2.02	5.96	2.32	7.13	1.60	13.58	3.67
T-bet+	0.48	0.06	4.74	2.00	2.92	2.54	2.57	0.48	12.02	4.19

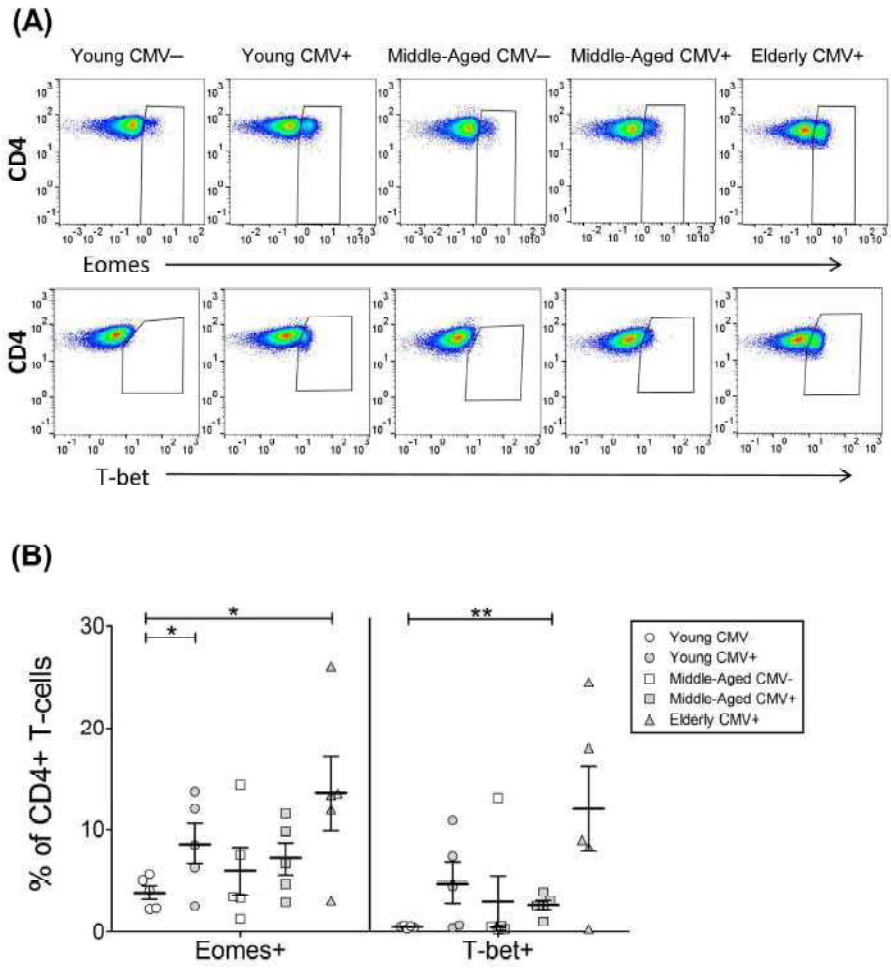

Figure 2. T-bet and Eomes expression on CD4+ T-cells. (**A**) Dot plot graphs representative of each group showing the expression of the studied markers on CD4+ T-cells; (**B**) percentage of CD4+ T-cells expressing T-bet and Eomes on healthy individuals (n = 25), stratified by age and CMV serostatus. Scatter plot shows the mean and SEM. Results were considered significant at * $p < 0.05$ and ** $p < 0.01$.

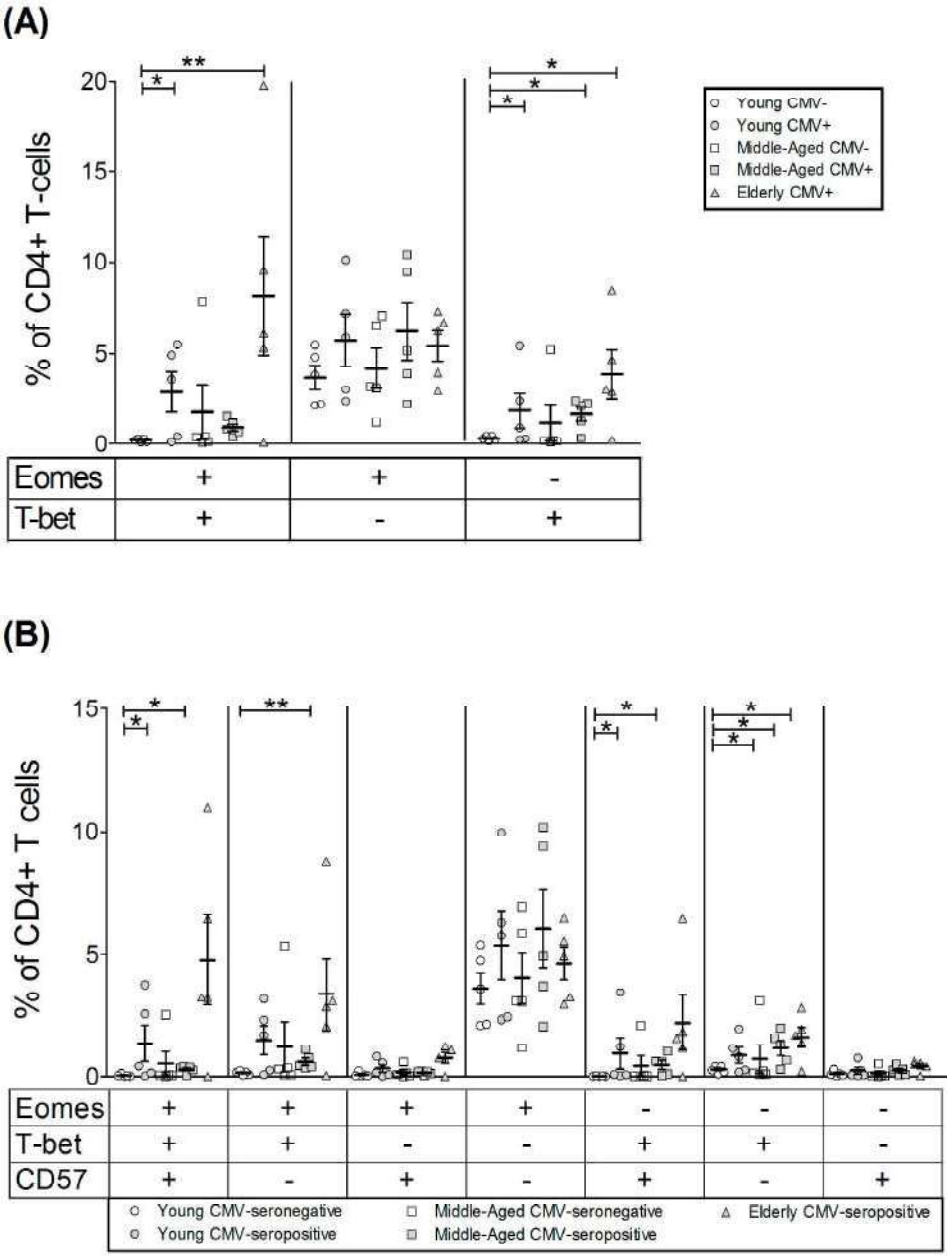

Figure 3. T-bet and Eomes coexpression with and without CD57 on CD4+ T-cells from healthy individuals (n = 25), stratified by age and CMV serostatus. (**A**) Coexpression of T-bet and Eomes on CD4+ T-cells; (**B**) T-bet, Eomes and CD57 coexpression on CD4+ T-cells. Scatter plots show the mean and SEM. The combination of markers studied is indicated in the table below the scatter graphs. Results were considered significant at * $p < 0.05$ and ** $p < 0.01$.

2.3. T-Bet and Eomes Expression on $CD4^{hi}CD8^{lo}$ T-Cells

The $CD4^{hi}CD8^{lo}$ subset was very low in young CMV-seronegative individuals, and, although there is no significant difference in percentage of cells, the individuals with the highest numbers of these cells were all CMV-seropositive, particularly the elderly (Figure S1). The percentage of $CD4^{hi}CD8^{lo}$ T-cells expressing Eomes was over 40% in all groups studied and noticeably higher than those of CD4+ T-cells (below 20%). Eomes+ $CD4^{hi}CD8^{lo}$ T-cells percentage was similar in all groups studied. The percentage of T-bet+ $CD4^{hi}CD8^{lo}$ T-cells was also higher than in CD4+ T-cells and particularly high in old CMV-seropositive individuals (Figure 4, Table 3).

Analysis of the coexpression pattern of Eomes and T-bet in $CD4^{hi}CD8^{lo}$ T-cell subset showed that the majority of cells from young CMV-seronegative individuals were Eomes+ T-bet− and the percentage of these cells were not affected by CMV infection or age. Cells expressing T-bet were present in significant numbers in the elderly CMV-seropositive individuals, especially the Eomes+ T-bet+ cells (Figure 5A).

The analysis of T-bet and Eomes expression in regards to CD57 expression on $CD4^{hi}CD8^{lo}$ T-cells (Figure 5B, Table S2) showed that the percentage of cells expressing both transcription factors and CD57 was very low in the young CMV-seronegative individuals and values were significantly higher in elderly seropositive donors. CD57+ $CD4^{hi}CD8^{lo}$ T-cells always coexpressed T-bet with or without Eomes. The majority of CD57− $CD4^{hi}CD8^{lo}$ T-cells were Eomes+ and were not affected by CMV infection. CD57−Eomes+T-bet+ cells increased with age in CMV-seropositive individuals.

Table 3. Percentage of $CD4^{hi}CD8^{lo}$ T-cells expressing T-bet and Eomes.

Transcription Factors	Group									
	Young CMV−		Young CMV+		Middle-Aged CMV−		Middle-Aged CMV+		Elderly CMV+	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
EOMES+	39.01	8.05	58.05	6.98	43.22	6.24	41.53	8.35	63.87	11.93
T-bet+	7.35	1.66	33.81	14.48	17.93	12.17	26.85	7.00	51.87	13.01

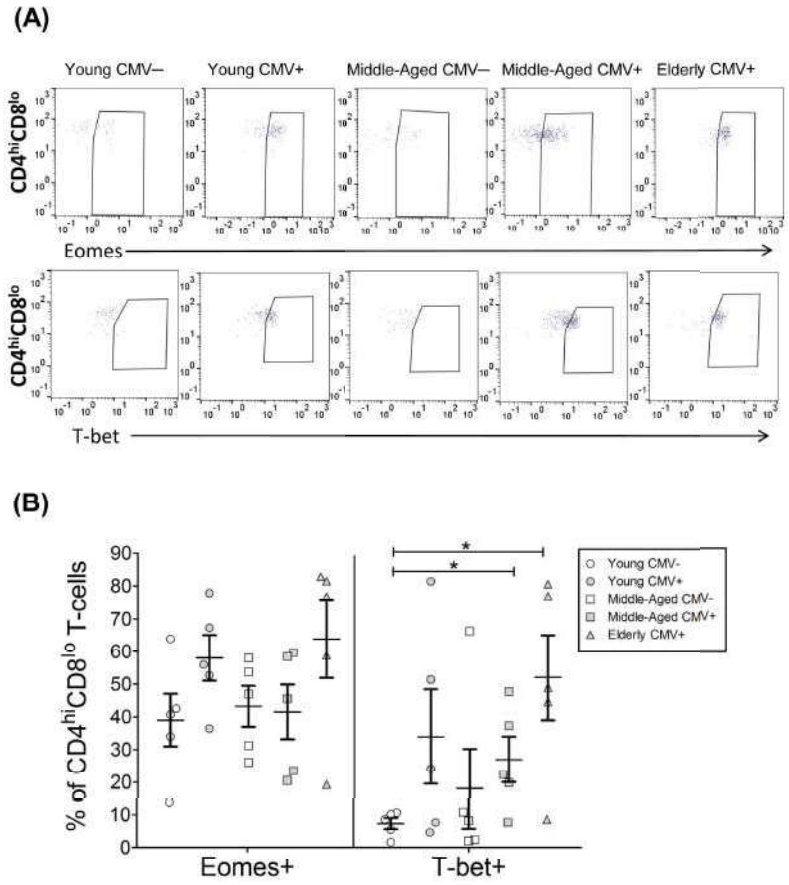

Figure 4. T-bet and Eomes expression on $CD4^{hi}CD8^{lo}$ T-cells. (**A**) Dot plot graphs show the expression of T-bet and Eomes on $CD4^{hi}CD8^{lo}$ T-cells for each group studied; (**B**) expression of T-bet and Eomes on $CD4^{hi}CD8^{lo}$ T-cells from healthy individuals (n = 25), stratified by age and CMV serostatus. Scatter plot shows the mean and SEM. Results were considered significant at * $p < 0.05$ and ** $p < 0.01$.

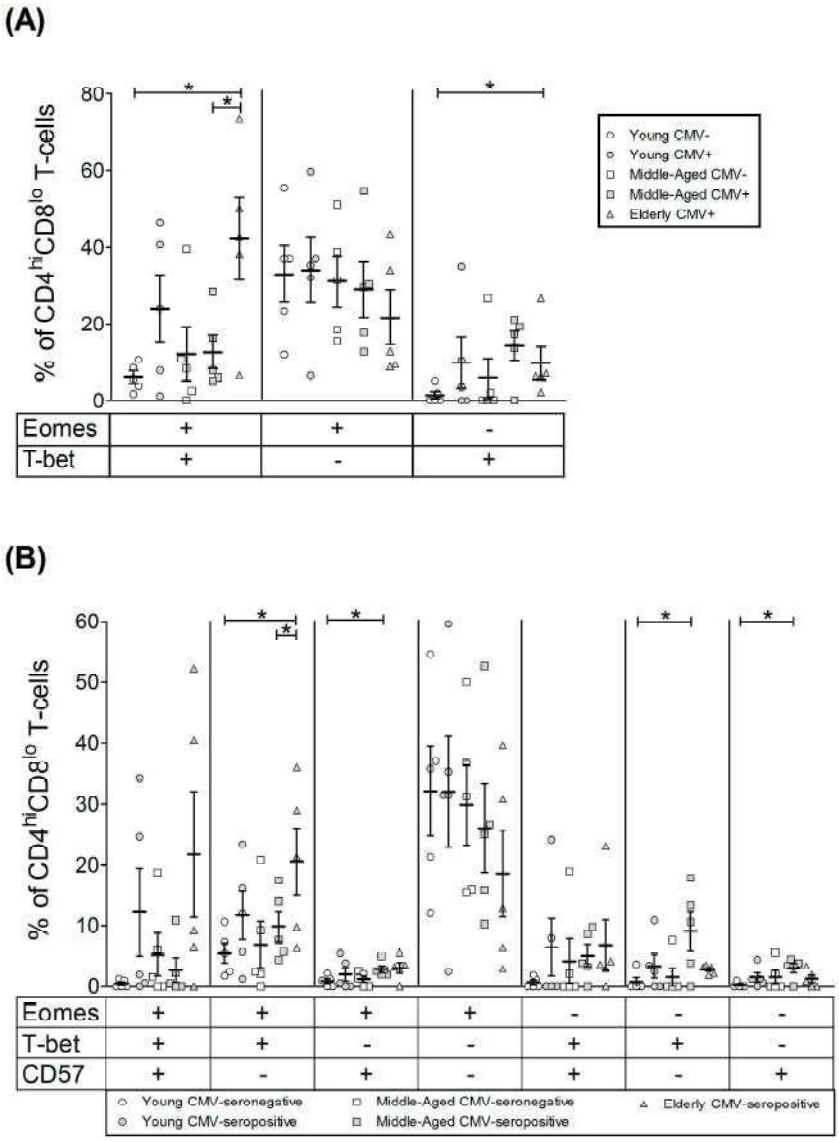

Figure 5. T-bet and Eomes coexpression with and without CD57 on $CD4^{hi}CD8^{lo}$ T-cells from healthy individuals (n = 25), stratified by age and CMV serostatus. (**A**) Coexpression of T-bet and Eomes on $CD4^{hi}CD8^{lo}$ T-cells; (**B**) T-bet, Eomes and CD57 Boolean combinations on $CD4^{hi}CD8^{lo}$ T-cells. Scatter plots show the mean and SEM. The combination of markers studied is indicated in the table below the scatter graphs. Results were considered significant at * $p < 0.05$ and ** $p < 0.01$.

2.4. T-Bet and Eomes Expression in CD8+ T-Cells

As expected, the percentage of CD8+ T-cells expressing T-bet and Eomes was higher than in CD4+ T-cells (Tables 3 and 4). The fraction of CD8+ T-cells expressing whichever transcription factor increased with age in CMV-seropositive individuals (Figure 6 and Figure S1, Table 4) Furthermore, the percentage of CD8+ T-cells that coexpressed both Eomes and T-bet was higher with age and reaching the highest values in the elderly CMV-seropositive individuals (Figure 7A).

The analysis according to CD57 expression (Figure 7B, Table S2) showed that the percentage of CD8+ T-cells coexpressing Eomes and T-bet increase with age, independently of CD57 expression. CD57+CD8+ T-cells expressed very low levels of Eomes in the absence of T-bet, although a proportion of CD57+CD8+ T-cells expressed T-bet without expressing Eomes. CD57+ CD8+ T-cells that did not express any of the transcription factors studied were very low or null in the elderly.

Table 4. Percentage of CD8+ T-cells expressing T-bet and Eomes.

Transcription Factors	Group									
	Young CMV−		Young CMV+		Middle-Aged CMV−		Middle-Aged CMV+		Elderly CMV+	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
EOMES+	39.28	4.57	46.09	3.54	49.58	6.57	55.73	4.42	63.24	3.31
T-bet+	29.60	3.18	29.28	5.91	40.43	8.46	57.30	6.17	62.64	10.51

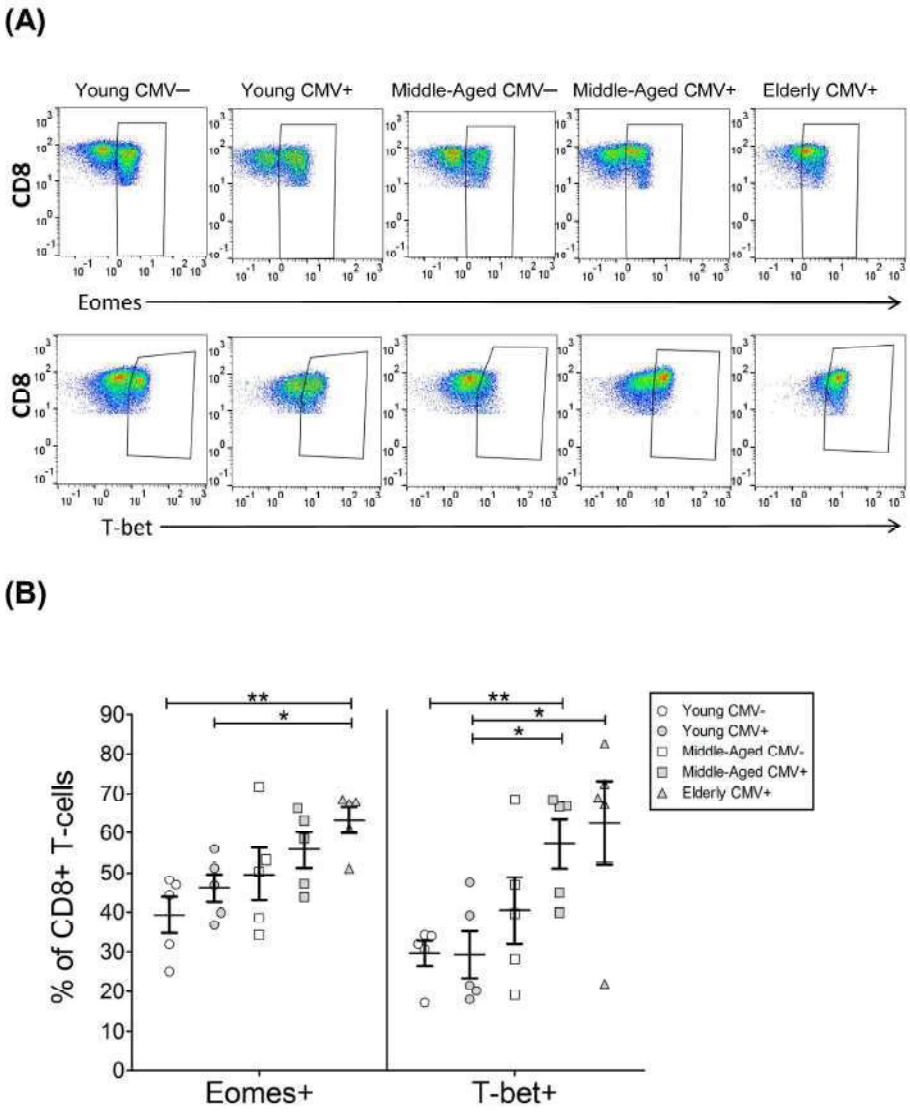

Figure 6. T-bet and Eomes expression on CD8+ T-cells. (**A**) Dot plot graphs representative of each group showing the expression of T-bet and Eomes on CD8+ T-cells; (**B**) T-bet and Eomes expression on CD8+ T-cells from healthy individuals (n = 25), stratified by age and CMV serostatus. Scatter plot shows the mean and SEM. Results were considered significant at * $p < 0.05$ and ** $p < 0.01$.

In summary, CD4+ T-cells expressing CD57 always coexpress T-bet and are only found in CMV-seropositive individuals independently of their age. In young CMV-seronegative individuals we did not observed expression of CD57 or T-bet by CD4+ T-cells. The expansion of these cells is clearly a hallmark of CMV infection and is further increased by age. Besides, $CD4^{hi}CD8^{lo}$ T-cells have a different phenotype than CD4+ T-cells, which do not express CD8. This subset contains higher numbers of

cells expressing CD57, T-bet and Eomes. These results support the hypothesis that $CD4^{hi}CD8^{lo}$ cells derive from CD4+ T-cells and are mature effector memory cells. On the other hand, the percentages of T-bet+CD8+ and Eomes+CD8+ T-cells were similar, increased with age and where higher than those of CD4+ T-cells. The majority of CD8+ T-cells coexpressed both transcription factors with or without CD57. Finally, in all subsets studied CD57+ T-cells were T-bet+ with or without Eomes but never Eomes+T-bet−.

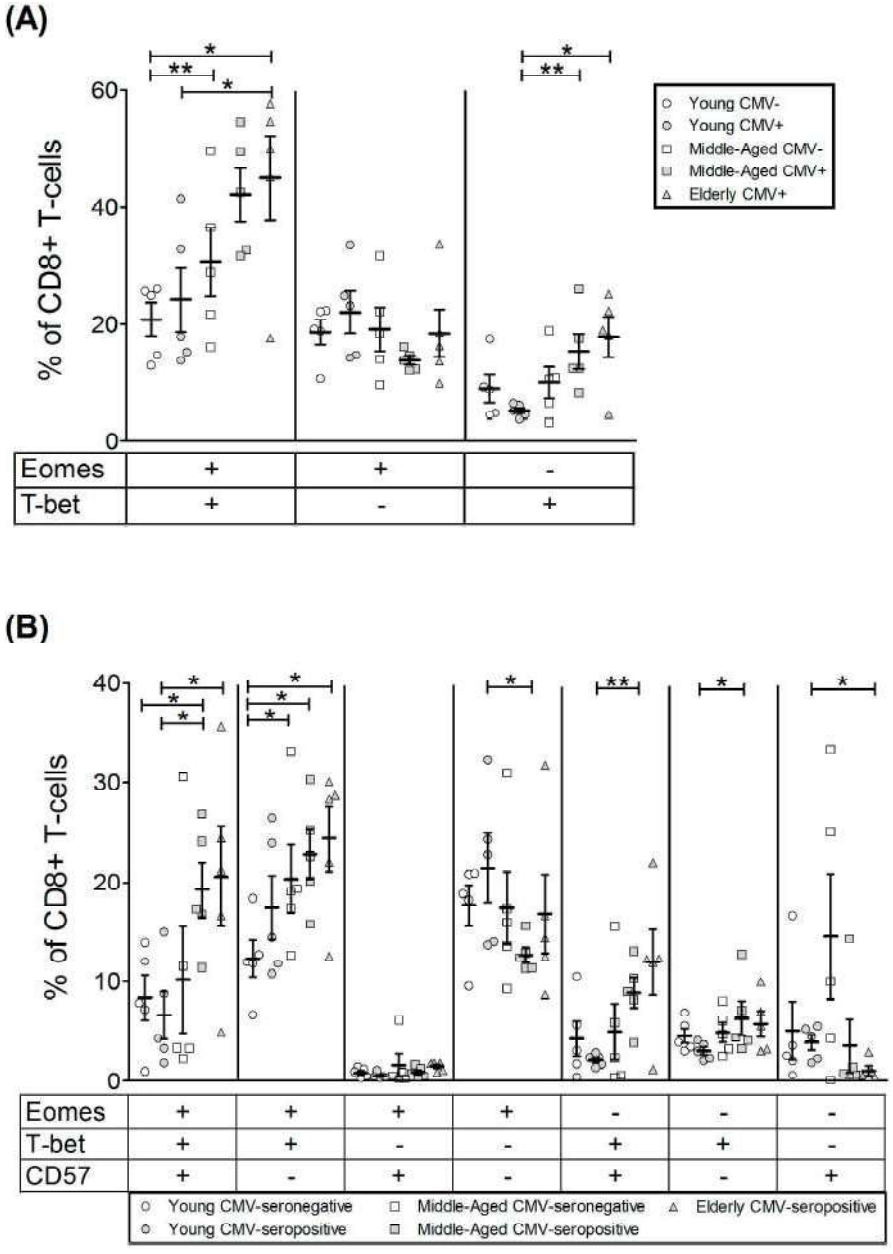

Figure 7. T-bet and Eomes coexpression with and without CD57 on CD8+ T-cells from healthy individuals (n = 25), stratified by age and CMV serostatus. (**A**) Coexpression of T-bet and Eomes on CD8+ T-cells; (**B**) T-bet, Eomes and CD57 coexpression on CD8+ T-cells. Scatter plots show the mean and SEM. The combination of markers studied is indicated in the table below the scatter graphs. Results were considered significant at * $p < 0.05$ and ** $p < 0.01$.

3. Discussion

Advances in the last decade on the processes involved in immunosenescence support the idea that some of the accepted hallmarks of T-cell immunosenescence in humans, such as the decreased numbers of peripheral naïve T-cells and the accumulations of memory T-cells (especially affecting the CD8+ T-cell compartment), are determined not only by the lower output of T-cells due to thymus involution but also by the individual's history of pathogen exposure, particularly to infection with CMV. Thus, it is generally accepted that CMV infection is a major driving force contributing to the age-associated deterioration of adaptive immunity [22–24]. One of these hallmarks of human immunosenescence associated with long-term CMV infection is the oligoclonal expansion of late-stage differentiated effector memory or effector T-cells, characterised by the downregulation of co-stimulatory molecules such as CD27 or CD28 and the expression of markers as KLRG-1 and CD57 [25].

CMV seropositivity increases with age in all the populations studied and is influenced by geographic, ethnic and socioeconomic factors [26] and CMV seropositivity has been associated with a significant increased risk of all-cause mortality [27].

Several studies have contributed to defining the expression of the transcription factors T-bet and/or Eomes in T-cells and their significance in controlling CD8+ and CD4+ T-cell functions in mice and human. In particular these transcription factors are involved in the development and maintenance of immune response to different pathogens [21,28].

In this study we characterise the expression of the transcription factors T-bet and Eomes in resting peripheral blood T-cell subsets defined by the expression of CD4, CD8 and CD57, from healthy individuals stratified according to age and CMV serostatus. Several studies have shown that T-bet and Eomes are expressed in human peripheral blood T-cells, with a higher expression on CD8+ than in CD4+ T-cells. In addition, whereas the percentages of naive cells CD4 and CD8 T-cells expressing T-bet or Eomes is very low, a high percentage of effector-memory and effector CD4 and CD8 T-cells express these factors [18].

It has been shown that human peripheral blood CD4+ T-cells are predominantly T-bet^{lo}Eomes$-$, suggesting that in peripheral blood CD4+ T-cells T-bet and Eomes likely do not significantly cooperate to modulate CD4+ T-cell function [18]. The levels of T-bet and Eomes are very low in CD4 naïve CD4 T-cells, and increase as T-cells become more differentiated and those CD4 T-cells that coexpress T-bet and Eomes show an effector or effector-memory phenotype [18]. Our results show that the percentage of resting CD4+ T-cells expressing T-bet T-cell was undetectable in young CMV-seronegative individuals whereas variable percentages of T-bet positive CD4+ T-cells were found in CMV-seropositive young donors, with higher values in the CMV-seropositive elderly individuals, supporting the relevant role of CMV in the generation of effector-memory and effector CD4+ T-cells. The expression of Eomes is also higher in CMV-seropositive (young and old) donors compared with CMV-seronegative young donors. Subsequently, the Boolean analysis of both transcription factors showed that the percentage of CD4+ T-cells coexpressing Eomes and T-bet cells was very low in young CMV-seronegative individuals and increased with CMV seropositivity in young and aged donors. The percentage of CD4+ T-cells expressing T-bet but not Eomes was also negligible in young CMV-seronegative individuals and increased in CMV-seropositive young, middle-aged and elderly donors. No significant changes were found in the percentages of CD4+ T-cells expressing Eomes but not T-bet among the groups studied.

The expression of CD57 on T-cells increases with age [29], and we have previously shown that whereas it is virtually absent on CD4 T-cells from CMV-seronegative young individuals, its expression is higher in CMV-seropositive donors [30]. Although CD57 was originally defined as a marker of senescent CD4 and CD8 T-cells, it is considered a marker of a subset of highly differentiated effector-memory and effector cells. Furthermore, in CMV-seropositive young individuals, CD57+CD4+ T-cells are polyfunctional cells and produce several cytokines, including IFNγ [30]. Since both T-bet and Eomes are known to induce IFNγ expression and other effector functions in CD4 T-cells, we next examined the coexpression of these factors according to the expression of CD57. As expected, no CD4

T-cells coexpressing CD57 and T-bet are found in young CMV-seronegative individuals. The majority of CD57+CD4+ T-cells that are found in the rest of groups studied express T-bet alone or in combination with Eomes, and CD57 expression is negligible in T-bet negative CD4 T-cells. It has been shown that stimulation of CD4 T-cells via TCR and CD28 results in the upregulation of T-bet in most CD4+ T-cells, whereas Eomes is only upregulated on a subset of polyfunctional CD4+ T-cells characterised by the expression of CD300a [31]. Since we have recently shown that CD57 is also a marker of polyfunctional CD4+ T-cells in CMV-seropositive young donors [30] and that the expression of CD300a on CD4+ T-cells is higher in CMV-seropositive aged donors [32], these results support the relevance of CMV infection on the expansion of effector memory and effector polyfunctional CD4+ T-cells that coexpress T-bet and Eomes. In a similar way, it has been shown that CD57+CD4+ T-cells that produce TNF-α and IFN-γ in response to CMV stimulation express higher levels of T-bet and Eomes when compared with CD57−CD4+ T-cells that produce TNF-α and express lower levels of both T-bet and Eomes [33]. The recent demonstration that T-bet induction on CD4+ T-cells in response to CMV stimulation, which correlated with proliferation and effector multifunction, allows us to differentiate lung transplant recipients mismatched for CMV that control CMV replication from those with early relapse [34] supports the relevance of this transcription factor in the generation and maintenance of CD4+ effector cells able to establish effective immune control during the early stages of CMV infection.

Peripheral blood double-positive (DP) CD4+ and CD8+ T-cells constitute a minor subset of T-cells that can be found in healthy individuals of different ages, and their percentage is increased in inflammatory autoimmune disease. An age-dependent increase in the different DP T-cell subsets described has been observed [35]. In particular, $CD4^{hi}CD8^{lo}$ (CD8$\alpha\alpha$) cells have been shown to be terminally differentiated effector CD4+ T-cells that acquire the alpha-chain of CD8 (for revision see [36]). Analysis of peripheral blood $CD4^{hi}CD8^{lo}$ T-cells has shown that these cells have a highly differentiated effector-memory phenotype, expressing more CXCR3 and CD57 than CD4+CD8− T-cells [37]. Our results show that the percentage of cells expressing both Eomes and T-bet was higher in the $CD4^{hi}CD8^{lo}$ subset than in CD4+CD8− T-cells and that the percentage of $CD4^{hi}CD8^{lo}$ T-cells expressing T-bet was associated with CMV-seropositivity. Similar to CD4+ T-cells, the coexpression of Eomes, T-bet and CD57 on $CD4^{hi}CD8^{lo}$ T-cells was mainly observed in CMV-seropositive donors. The analysis of T-bet and Eomes on $CD4^{hi}CD8^{lo}$ T-cells supports the notion that these cells represent a subset of highly differentiated CD4+ T-cells that are significantly expanded in CMV-seropositive elderly individuals.

We have also analysed T-bet and Eomes in CD8+ T-cells in relation to the expression of CD57. It has been described that CD57 is expressed in a significant percentage of CD8+ T-cells that increases with aging, in particular in CMV-seropositive donors, and has been considered a marker of dysfunctional senescent T-cells [38–43]. However, as indicated above for CD57+CD4+ T-cells, recent analysis of its expression in healthy young individuals supports the fact that CD57+CD8+ T-cells represent a subset of effector-memory and effector cells that are polyfunctional cells able to produce cytokines, including IFNγ [44].

It has also been shown that high levels of T-bet in CD8+ T-cells are associated with long-term resilience, low expression of inhibitory receptors, and protection from exhaustion in the experimental model of murine Lymphocytic Choriomeningitis Virus (LCMV) infection [19] and in elite non-progressor HIV-infected patients [20]. It has been proposed that in chronic viral infection, such as murine LCMV infection or human hepatitis C virus (HCV), CD8+ cells expressing $T\text{-}bet^{hi}$ proliferate in response to persisting antigen, leading to $Eomes^{hi}$ terminal differentiation [21] and supporting the relevance of these transcription factors in the maintenance of antiviral CD8+ T-cells during chronic viral infection. The analysis of T-bet and Eomes expression in CD8+ T-cells according to CMV-serostatus and age shows that the percentage of CD8+ T-cells positive for any of the transcription factors increases from CMV-seronegative young individuals to CMV-seropositive old donors. In particular, the percentage of CD8+ T-cells expressing T-bet but not Eomesis is higher in middle-aged and elderly CMV-seropositive donors compared with young CMV-seropositive individuals, whereas the percentage of those CD8+

T-cells coexpressing T-bet and Eomes is also higher in middle-aged CMV-seronegative compared with young CMV-seronegative donors. Higher percentages of CD8+ T-cells coexpressing T-bet and Eomes observed in old CMV-seropositive donors are found in both CD57−CD8+ and CD57+CD8+ T-cell subsets. No changes are observed in the percentage of Eomes single-positive CD8 cells (without T-bet) among the different groups considered. The majority of these cells do not express CD57 as the percentage of Eomes+CD57+CD8+ T-cells is negligible. These findings are in agreement with the recent observation showing that the majority (60–70%) of NKT-like effector CD8+ T-cells, defined by the expression of CD56, CD57 CD45RA, CD49d and KIR/NKG2A inhibitory receptors, coexpress Eomes and T-bet [45].

Two different subsets of CMV-specific CD8+ T-cells have been defined according to the expression of T-bet and Eomes [28]. Those expressing high levels of T-bet and lower levels of Eomes are the dominant populations and are highly efficient at the recognition of endogenously processed peptide–MHC complexes, although they show a low avidity for peptide–MHC, whereas those cells with lower levels of T-bet and high levels of Eomes represent the subdominant populations, are less efficient in the recognition of virus-infected cells, and have high peptide-MHC avidity [28]. Studies in mice have shown that the continuous stimulation of CMV-specific T-cells by persistent antigen exposure is required to maintain functional effector CD8+ T-cells responsible for protection against viral reactivation [46,47]. This sustained antigen exposure throughout life likely contributes to the age-associated oligoclonal expansion of two different subsets of CD8+ T-cells coexpressing graded levels of T-bet and of Eomes and reflects a balance between short-lived terminal CD57+ effector CD8+ T-cells and long-lived CD57− effector-memory CD8+ lymphocytes, characteristic of the periodic replenishment of specific T-cells necessary to achieve lifelong CMV immunity, as has been recently postulated for chronic infections [21,28].

The analysis of T-bet and Eomes in CD8 T-cells obtained from lung transplant recipients mismatched for CMV serostatus showed that patients showing higher ratios of T-bet:Eomes expression in CD8+ T-cells are able to control CMV replication versus with those with lower ratios that were viremic relapsers. CD8+ T-cells showing high ratios of T-bet:Eomes are better responders to CMV stimulation in terms of proliferation and, cytotoxicity and cytokine production, underscoring the importance of the T-bet:Eomes balance, with CMV-specific proliferation a key factor driving early T-bet expression and effector function in CD8+ T-cells during primary infection to establish immune control during early stages of CMV chronic infection [48].

In summary, our results support the relevance of CMV and aging in the expansion of highly differentiated CD4+ and CD8+ T-cells that differentially express T-bet and/or Eomes transcription factors. The expansion of these cells found in CMV-seropositive elderly donors suggest that the expression of these factors is essential not only for the development but also for the maintenance of an adequate pool of effector-memory and effector CD4+ and CD8+ T lymphocytes required to achieve lifelong immunity against chronic CMV infection. Further studies are required to define the impact of CMV and ageing on the expression of T-bet and Eomes in other T-cell subpopulations, such as the well-defined central memory, effector-memory or effector subsets, and the relevance of these transcription factors in the differentiation process of T-cells involved in the response to viruses and other pathogens.

4. Materials and Methods

4.1. Subjects

We studied 25 healthy donors stratified according to age and CMV serostatus (Table 1). However, all elderly donors included in the study were CMV-seropositive, as we were not able to recruit enough CMV-seronegative individuals given that the prevalence of CMV seropositivity in Spain in individuals over the age of 40 years is 80% [49] and, in our geographic area (Andalusia, Spain), about 99% of individuals over 65 years are CMV-seropositive.

All study participants provided informed written consent and met the following exclusion criteria: absence of diabetes, cancer, severe renal failure, severe liver disease, endocrine disorders, autoimmune diseases, or acute infectious disease; and they were not consuming drugs whose activity is known to modify the functions of the immune system. The ethical statement was approved by the Ethics Committee of the Reina Sofia University Hospital of Cordoba (Spain), on 25 February 2014 (reference number 2465). Peripheral blood from each subject was collected in lithium heparin tubes, followed by PBMCs isolation by density gradient centrifugation using Ficoll Histopage-1077 (Sigma-Aldrich, St. Louis, MO, USA). After isolation PBMCs were cryopreserved in FBS (Sigma-Aldrich) with 10% DMSO (Panreac Chemistry SAU, Barcelona, Spain) until experiments were performed.

4.2. CMV Serology

CMV-specific IgG and IgM was determined by automated enzyme-linked immunosorbent assay (ELISA) (DRG International, Mountainside, NY, USA) from plasma retrieved from all donors.

4.3. Flow Cytometry and Data Analysis

For cytometry experiments, cells were thawed in RPMI 1640 (Sigma-Aldrich) with 10% FBS (Gibco Life Technologies, Carlsbad, CA, USA) and subsequently placed in a 96-well plate at 2×10^6 cells/mL concentration (250 µL final volume). Cells were then washed twice with PBS (4 °C) and surface stained for the following antibodies: anti-Live/Dead APC-Vio770 (LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, life technologies), anti-CD3 VioBlue (clone: BW264/56, MiltenyiBiotec, BergischGladbach, Germany), anti-CD57 Biotin-Anti-Biotin-Viogreen (clone: TB03, MiltenyiBiotec), anti-CD8 APC (clone: BW135/80, MiltenyiBiotec) and anti-CD4 PE-Vio770 (clone: M-T466, MiltenyiBiotec). Following cell fixation and permeabilisation using the Kit FoxP3 Staining Buffer Set (MiltenyiBiotec), intracellular staining of the transcription factors was performed according to the manufacturer's instructions, with the following antibodies: anti-T-bet PerCP Cy5.5 (clone: 04-46, BD Pharmingen, San Diego, CA, USA) and anti-Eomes FITC (clone: WD1928, eBioscience, Waltham, MA, USA). All antibodies were titrated before use.

Samples were then acquired with a nine-parameter MACsQuant instrument (MiltenyiBiotech, BergischGladbach, Germany) and analysed with FlowJo v X 10.0.7 software (TreeStar, Portland, OR, USA). For data analysis, lymphocytes were gated according to their size and granularity (FSC vs. SSC), then forward scatter height (FSC-H) versus forward scatter area (FSC-A) to remove doublets. Within the singlets gate, Live T-cells (CD3+) were gated, followed by identification of the different T-cell subsets by confronting CD4 vs. CD8. Individual gates for T-bet+, Eomes+ and CD57+ T-cells were gated on each of these populations based on fluorescence minus one controls. A representative example of the gating strategy is shown in Figure 1. FlowJo's Boolean combination gating options were used to analyse the coexpression of T-bet and Eomes and according to CD57 expression as well.

4.4. Statistical Analysis

Data were inspected for normal distribution using the Shapiro–Wilk test. No normality was found. The Mann–Whitney U nonparametric test was used to derive p-values for comparing data among the specific sample pairs. All statistical tests were performed with PASW Statistics v. 18. For graphs, GraphPad Prism (version 5.0, GraphPad Software, Inc., La Jolla, CA, USA) was used. Results are represented as scatter plots including the mean and SEM. The median and interquartile ranges of the coexpression analysis of the transcription factor T-bet and Eomes and CD57 on T-cell subsets is shown in the Supplementary Materials (Tables S1 and S2).

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/18/7/1391/s1.

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Author Contributions: Rafael Solana, Alejandra Pera and Raquel Tarazona designed the study. Fakhri Hassouneh collected the data and performed the laboratory experiments. Fakhri Hassouneh and Nelson Lopez-Sejas collaborated in the laboratory analysis. Alejandra Pera and Fakhri Hassouneh performed the statistical analysis and wrote the draft. Raquel Tarazona, Beatriz Sanchez-Correa and Carmen Campos made significant technical and conceptual contributions to the manuscript. Rafael Solana, Raquel Tarazona, and Alejandra Pera reviewed the final version of the paper. All the authors provided intellectual content and approved the final version of the paper. Rafael Solana and Alejandra Pera are co-senior authors and have contributed equally to this work.

Conflicts of Interest: The authors declare no conflict of interest.

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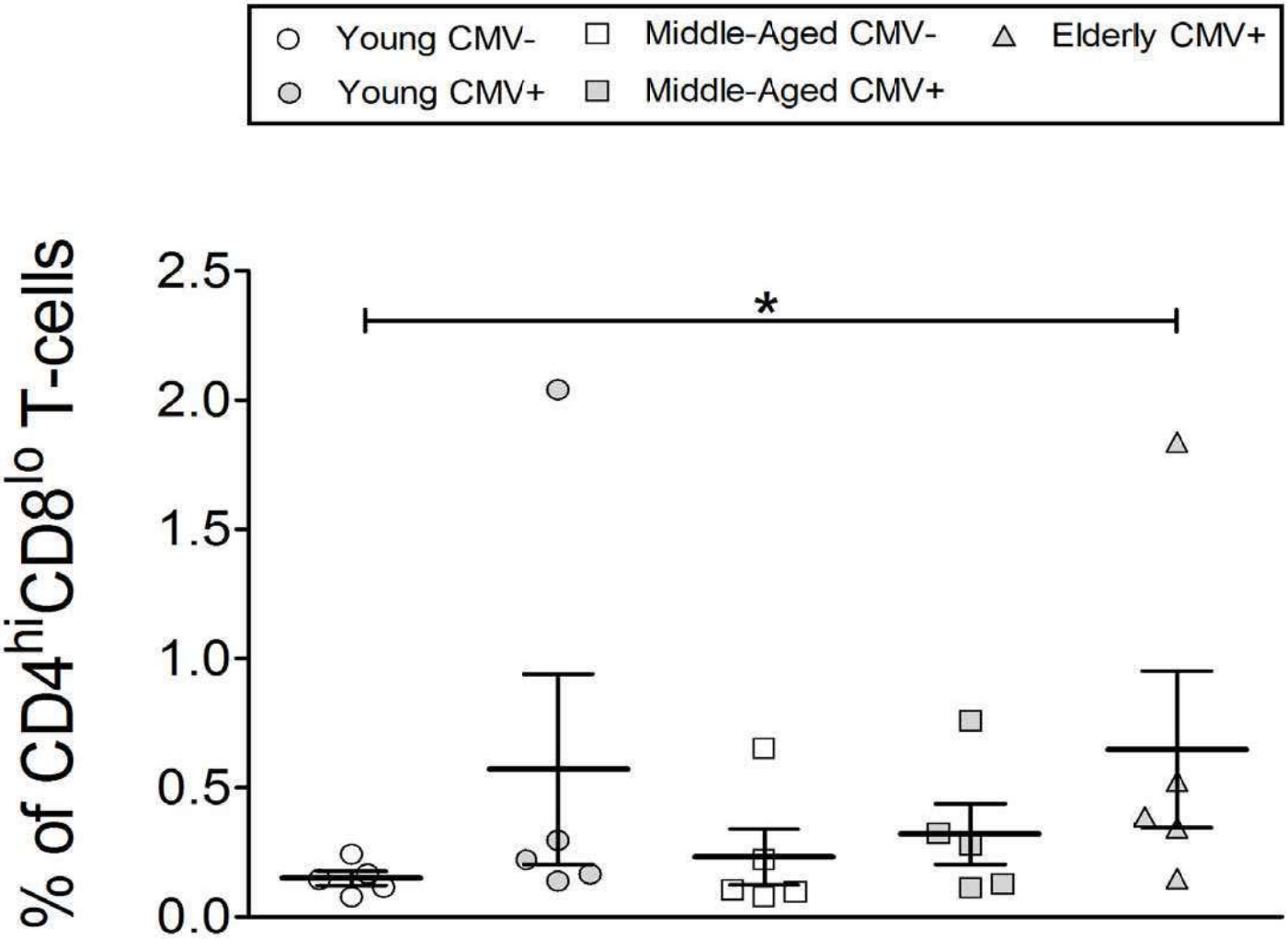

Figure S1. Percentage of $CD4^{hi}CD8^{lo}$ T-cells. Graph shows the percentage of $CD4^{hi}CD8^{lo}$ T-cells from healthy individuals (n=25), stratified by age and CMV serostatus. Scatter plot showing the mean and SEM. Results were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Table S1. Median and InterQuartile Range (IQR) of the Co-expression analysis of T-bet and Eomes on T-cell subsets. A) CD4 and $CD4^{hi}CD8^{lo}$ phenotype

CMV status	CD4+ T-cells	Median	Percentile 25	Percentile 75	$CD4^{hi}CD8^{lo}$	Median	Percentile 25	Percentile 75
Young CMV-seronegative	CD4+EOMES+	4,13	2,28	5,07	$CD4^{hi}CD8^{lo}$ EOMES+	40,74	34,04	42,59
	CD4+T-bet+	,51	,43	,55	$CD4^{hi}CD8^{lo}$ T-bet+	8,64	5,56	10,19
	CD4+EOMES+T-bet+	,18	,08	,28	$CD4^{hi}CD8^{lo}$ EOMES+T-bet+	5,56	3,70	8,33
	CD4+EOMES+T-bet-	3,83	2,19	4,79	$CD4^{hi}CD8^{lo}$ EOMES+T-bet-	37,04	23,40	37,04
	CD4+EOMES-T-bet+	,27	,20	,44	$CD4^{hi}CD8^{lo}$ EOMES-T-bet+	,00	,00	1,85
					$CD4^{hi}CD8^{lo}$ cells	,15	,12	,17
Young CMV-seropositive	CD4+EOMES+	8,51	6,26	12,04	$CD4^{hi}CD8^{lo}$ EOMES+	56,00	52,70	67,31
	CD4+T-bet+	4,49	,64	7,29	$CD4^{hi}CD8^{lo}$ T-bet+	24,00	7,69	51,23
	CD4+EOMES+T-bet+	3,59	,39	4,90	$CD4^{hi}CD8^{lo}$ EOMES+T-bet+	24,00	7,69	40,74
	CD4+EOMES+T-bet-	5,86	3,03	7,14	$CD4^{hi}CD8^{lo}$ EOMES+T-bet-	35,29	32,00	37,04
	CD4+EOMES-T-bet+	,90	,29	2,39	$CD4^{hi}CD8^{lo}$ EOMES-T-bet+	3,53	,00	10,49
					$CD4^{hi}CD8^{lo}$ cells	,22	,17	,30
Middle-Aged CMV-seronegative	CD4+EOMES+	3,46	3,29	7,43	$CD4^{hi}CD8^{lo}$ EOMES+	46,94	31,25	53,75
	CD4+T-bet+	,48	,26	,58	$CD4^{hi}CD8^{lo}$ T-bet+	8,16	2,50	10,77
	CD4+EOMES+T-bet+	,36	,10	,40	$CD4^{hi}CD8^{lo}$ EOMES+T-bet+	8,16	2,50	10,77
	CD4+EOMES+T-bet-	3,19	3,10	6,48	$CD4^{hi}CD8^{lo}$ EOMES+T-bet-	31,25	18,51	38,78
	CD4+EOMES-T-bet+	,16	,14	,22	$CD4^{hi}CD8^{lo}$ EOMES-T-bet+	,00	,00	2,08
					$CD4^{hi}CD8^{lo}$ cells	,11	,10	,22
Middle-Aged CMV-seropositive	CD4+EOMES+	6,69	4,70	9,81	$CD4^{hi}CD8^{lo}$ EOMES+	45,56	23,53	58,45
	CD4+T-bet+	2,48	2,45	3,03	$CD4^{hi}CD8^{lo}$ T-bet+	22,17	19,61	37,22
	CD4+EOMES+T-bet+	,81	,67	1,18	$CD4^{hi}CD8^{lo}$ EOMES+T-bet+	7,69	5,88	16,11
	CD4+EOMES+T-bet-	5,16	3,89	9,45	$CD4^{hi}CD8^{lo}$ EOMES+T-bet-	29,44	17,65	30,19
	CD4+EOMES-T-bet+	2,12	1,27	2,22	$CD4^{hi}CD8^{lo}$ EOMES-T-bet+	17,24	13,73	19,32
					$CD4^{hi}CD8^{lo}$ cells	,28	,13	,32
Elderly CMV-seropositive	CD4+EOMES+	13,37	11,97	13,51	$CD4^{hi}CD8^{lo}$ EOMES+	76,78	58,87	81,49
	CD4+T-bet+	9,01	8,33	18,01	$CD4^{hi}CD8^{lo}$ T-bet+	48,82	44,48	76,88
	CD4+EOMES+T-bet+	6,09	5,31	9,55	$CD4^{hi}CD8^{lo}$ EOMES+T-bet+	42,65	38,21	50,21
	CD4+EOMES+T-bet-	6,23	3,95	6,66	$CD4^{hi}CD8^{lo}$ EOMES+T-bet-	12,90	9,26	34,12
	CD4+EOMES-T-bet+	3,02	2,92	4,68	$CD4^{hi}CD8^{lo}$ EOMES-T-bet+	6,27	6,16	6,94
					$CD4^{hi}CD8^{lo}$ cells	,39	,34	,52

Table S1. Median and InterQuartile Range (IQR) of the Co-expression analysis of T-bet and Eomes on T-cell subsets. B) CD8 phenotype				
CMV status	**CD8+ T-cells**	Median	Percentile 25	Percentile 75
Young CMV-seronegative	CD8+EOMES+	44,25	31,91	46,91
	CD8+T-bet+	31,98	30,69	33,94
	CD8+EOMES+T-bet+	24,78	14,58	25,56
	CD8+EOMES+T-bet-	19,10	18,69	22,13
	CD8+EOMES-T-bet+	8,67	4,77	9,16
Young CMV-seropositive	CD8+EOMES+	46,81	39,82	51,21
	CD8+T-bet+	21,53	20,22	39,17
	CD8+EOMES+T-bet+	17,75	15,10	32,68
	CD8+EOMES+T-bet-	23,06	14,54	24,72
	CD8+EOMES-T-bet+	5,12	4,36	6,12
Middle-Aged CMV-seronegative	CD8+EOMES+	50,28	38,45	53,20
	CD8+T-bet+	39,58	28,16	46,84
	CD8+EOMES+T-bet+	28,93	21,64	36,47
	CD8+EOMES+T-bet-	18,24	13,81	22,11
	CD8+EOMES-T-bet+	10,37	6,51	10,65
Middle-Aged CMV-seropositive	CD8+EOMES+	58,55	47,10	63,06
	CD8+T-bet+	66,64	44,82	66,85
	CD8+EOMES+T-bet+	42,50	32,55	49,42
	CD8+EOMES+T-bet-	13,64	12,13	14,55
	CD8+EOMES-T-bet+	12,28	12,27	17,42
Elderly CMV-seropositive	CD8+EOMES+	67,47	61,23	67,93
	CD8+T-bet+	68,87	67,27	72,45
	CD8+EOMES+T-bet+	49,98	45,03	54,48
	CD8+EOMES+T-bet-	16,20	13,45	18,49
	CD8+EOMES-T-bet+	18,89	17,97	22,24

Table S2. Median and InterQuartile Range (IQR) of the Co-expression analysis of T-bet, Eomes and CD57 on T-cell subsets A) CD4 and $CD4^{hi}CD8^{lo}$ phenotype

CMV status	CD4+ T-cells	Median	Percentile 25	Percentile 75	$CD4^{hi}CD8^{lo}$	Median	Percentile 25	Percentile 75
Young CMV-seronegative	CD4+Eomes+T-bet+CD57+	,02	,01	,03	$CD4^{hi}CD8^{lo}$ Eomes+T-bet+CD57+	,00	,00	,93
	CD4+Eomes+Tbet+CD57-	,17	,07	,17	$CD4^{hi}CD8^{lo}$ Eomes+Tbet+CD57-	5,56	2,47	7,41
	CD4+Eomes+Tbet-CD57+	,03	,02	,06	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57+	,93	,00	1,23
	CD4+Eomes+Tbet-CD57-	3,58	2,16	4,77	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57-	35,80	21,28	37,04
	CD4+Eomes-Tbet+CD57+	,01	,01	,02	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57+	,00	,00	1,23
	CD4+Eomes-Tbet+CD57-	,26	,19	,43	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57-	,00	,00	,00
	CD4+Eomes-Tbet-CD57+	,08	,05	,11	$CD4^{hi}CD8^{lo}$ Eomes-Tbet-CD57+	,00	,00	,00
Young CMV-seropositive	CD4+Eomes+T-bet+CD57+	,43	,14	2,57	$CD4^{hi}CD8^{lo}$ Eomes+T-bet+CD57+	1,92	,57	24,69
	CD4+Eomes+Tbet+CD57-	1,73	,25	2,33	$CD4^{hi}CD8^{lo}$ Eomes+Tbet+CD57-	12,05	5,77	16,05
	CD4+Eomes+Tbet-CD57+	,17	,08	,57	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57+	,57	,00	3,90
	CD4+Eomes+Tbet-CD57-	5,78	2,46	6,31	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57-	31,48	31,43	35,29
	CD4+Eomes-Tbet+CD57+	,09	,06	1,23	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57+	,00	,00	8,02
	CD4+Eomes-Tbet+CD57-	,81	,27	1,17	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57-	2,47	,00	3,53
	CD4+Eomes-Tbet-CD57+	,06	,05	,25	$CD4^{hi}CD8^{lo}$ Eomes-Tbet-CD57+	1,14	,62	1,18
Middle-Aged CMV-seronegative	CD4+Eomes+T-bet+CD57+	,05	,05	,06	$CD4^{hi}CD8^{lo}$ Eomes+T-bet+CD57+	1,54	,00	6,12
	CD4+Eomes+Tbet+CD57-	,30	,06	,35	$CD4^{hi}CD8^{lo}$ Eomes+Tbet+CD57-	2,50	2,04	9,23
	CD4+Eomes+Tbet-CD57+	,09	,03	,13	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57+	1,25	,00	2,04
	CD4+Eomes+Tbet-CD57-	3,07	3,06	5,87	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57-	31,25	15,80	36,73
	CD4+Eomes-Tbet+CD57+	,01	,01	,04	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57+	,00	,00	2,08
	CD4+Eomes-Tbet+CD57-	,15	,10	,21	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57-	,00	,00	,00
	CD4+Eomes-Tbet-CD57+	,03	,03	,06	$CD4^{hi}CD8^{lo}$ Eomes-Tbet-CD57+	,00	,00	2,08
Middle-Aged CMV-seropositive	CD4+Eomes+T-bet+CD57+	,37	,26	,39	$CD4^{hi}CD8^{lo}$ Eomes+T-bet+CD57+	,49	,00	2,22
	CD4+Eomes+Tbet+CD57-	,44	,40	,78	$CD4^{hi}CD8^{lo}$ Eomes+Tbet+CD57-	7,69	5,88	13,89
	CD4+Eomes+Tbet-CD57+	,20	,13	,20	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57+	2,56	1,97	2,78
	CD4+Eomes+Tbet-CD57-	4,96	3,69	9,42	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57-	25,12	15,69	26,67
	CD4+Eomes-Tbet+CD57+	,60	,10	,63	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57+	3,94	3,33	8,70
	CD4+Eomes-Tbet+CD57-	1,32	,67	1,60	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57-	10,63	3,92	13,30
	CD4+Eomes-Tbet-CD57+	,27	,09	,30	$CD4^{hi}CD8^{lo}$ Eomes-Tbet-CD57+	3,45	3,33	3,92
Elderly CMV-seropositive	CD4+Eomes+T-bet+CD57+	3,22	3,22	6,47	$CD4^{hi}CD8^{lo}$ Eomes+T-bet+CD57+	9,25	6,64	40,43
	CD4+Eomes+Tbet+CD57-	2,87	2,10	3,09	$CD4^{hi}CD8^{lo}$ Eomes+Tbet+CD57-	21,30	9,79	28,96
	CD4+Eomes+Tbet-CD57+	,78	,72	1,10	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57+	3,32	2,78	3,58
	CD4+Eomes+Tbet-CD57-	4,97	3,24	5,56	$CD4^{hi}CD8^{lo}$ Eomes+Tbet-CD57-	12,90	6,48	30,81
	CD4+Eomes-Tbet+CD57+	1,60	1,19	1,87	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57+	3,70	2,99	4,27
	CD4+Eomes-Tbet+CD57-	1,72	1,42	1,98	$CD4^{hi}CD8^{lo}$ Eomes-Tbet+CD57-	3,24	2,15	3,28
	CD4+Eomes-Tbet-CD57+	,50	,42	,56	$CD4^{hi}CD8^{lo}$ Eomes-Tbet-CD57+	,90	,00	1,90

Table S2. Median and InterQuartile Range (IQR) of the Co-expression analysis of T-bet, Eomes and CD57 on T-cell subsets. B) CD8 phenotype

CMV status	CD8+ T-cells	Median	Percentile 25	Percentile 75
Young CMV-seronegative	CD8+Eomes+T-bet+CD57+	7,87	7,20	12,03
	CD8+Eomes+Tbet+CD57-	11,98	11,81	12,76
	CD8+Eomes+Tbet-CD57+	,96	,51	1,21
	CD8+Eomes+Tbet-CD57-	18,81	18,18	20,86
	CD8+Eomes-Tbet+CD57+	3,09	1,74	5,70
	CD8+Eomes-Tbet+CD57-	3,92	3,46	5,59
	CD8+Eomes-Tbet-CD57+	2,56	2,04	3,57
Young CMV-seropositive	CD8+Eomes-Tbet-CD57-	4,31	3,30	8,79
	CD8+Eomes+T-bet+CD57+	14,45	11,81	23,89
	CD8+Eomes+Tbet+CD57-	,45	,42	,50
	CD8+Eomes+Tbet-CD57+	22,77	14,04	24,27
	CD8+Eomes+Tbet-CD57-	2,35	1,70	2,43
	CD8+Eomes-Tbet+CD57+	2,97	2,29	3,68
	CD8+Eomes-Tbet+CD57-	4,55	2,27	5,26
Middle-Aged CMV-seronegative	CD8+Eomes-Tbet-CD57+	3,33	3,31	11,52
	CD8+Eomes-Tbet-CD57-	19,07	17,41	19,38
	CD8+Eomes+T-bet+CD57+	,56	,22	,90
	CD8+Eomes+Tbet+CD57-	15,96	13,59	17,34
	CD8+Eomes+Tbet-CD57+	2,36	,60	5,92
	CD8+Eomes+Tbet-CD57-	4,73	3,23	6,09
	CD8+Eomes-Tbet+CD57+	10,00	4,35	25,00
Middle-Aged CMV-seropositive	CD8+Eomes-Tbet+CD57-	17,31	16,77	24,07
	CD8+Eomes-Tbet-CD57+	22,61	20,16	25,18
	CD8+Eomes-Tbet-CD57-	,83	,50	1,25
	CD8+Eomes+T-bet+CD57+	12,39	11,37	12,92
	CD8+Eomes+Tbet+CD57-	8,98	8,19	10,32
	CD8+Eomes+Tbet-CD57+	4,39	4,09	7,10
	CD8+Eomes+Tbet-CD57-	,79	,69	1,41
Elderly CMV-seropositive	CD8+Eomes-Tbet+CD57+	21,27	16,68	24,39
	CD8+Eomes-Tbet+CD57-	28,35	22,07	28,71
	CD8+Eomes-Tbet-CD57+	1,78	1,14	1,82
	CD8+Eomes-Tbet-CD57-	14,37	12,54	16,67
	CD8+Eomes+T-bet+CD57+	12,28	11,89	12,36
	CD8+Eomes+Tbet+CD57-	5,61	3,24	7,01
	CD8+Eomes+Tbet-CD57+	,58	,44	,98

5. GENERAL DISCUSSION

Our work demonstrates that the effect of CMV varies with time, highlighting the importance of taking into account both age and CMV status in any study regarding the immune system. Specifically, here we show how NKT-like cells do not expand with CMV alone but only accumulate with age in CMV-seropositive individuals. Additionally, our results confirm that CD57 is not only a polyfunctional marker of CD4+ and CD8+T-cells (25, 26), but also NKT cells, suggesting that any T-cell expressing this marker will be a polyfunctional effector cell, key for the immune response against virus infection. As life spans these cells may become a potential danger as are capable of producing high amounts of pro-inflammatory cytokines.

Besides, our analysis has shown that the effect of CMV on CD300a and CD161 receptors depends on the age of the individual and changes with the type of T-cell. All cells co-expressing both CD57 and CD300a are polyfunctional (25, 26, 108) pointing out these markers as important for the host defense against virus infection. However, we still have to answer whether CD300a+ T-cells that do not express CD57 have the same functional capacities as the CD300a+CD57+. On the other hand, CD161 which has been associated with IL-17 production (116) decreases with age in CMV-seropositive individuals. This result indicates that long term infection with CMV could dampen the IL-17 response. Future investigations should address if age alone is capable of diminishing CD161 expression as well.

Finally, our work has shown that CMV infection in young individuals increases the expression levels of T-bet and Eomes in CD4+ T-cells. This supports the relevant role of CMV in the generation of effector-memory and effector CD4+ T-cell (134, 135). Therefore, no CD4+ T-cells co-expressing CD57 and T-bet are found in young CMV-seronegative individuals. Thus, the expansion of T-bet+CD57+ CD4+ T-cells is clearly a hallmark of CMV infection and is further increased by age. Besides, $CD4^{hi}CD8^{lo}$ T-cells have a different phenotype than CD4+ T-cells, which do not express CD8 (28). This subset contains higher numbers of cells expressing CD57, T-bet and Eomes. These

results support the hypothesis that $CD4^{hi}CD8^{lo}$ cells derive from CD4+ T-cells and are mature effector memory cells (29). On the other hand, the percentages of T-bet+CD8+ and Eomes+CD8+ T-cells were similar, increased with age and where higher than those of CD4+ T-cells. The majority of CD8+ T-cells co-expressed both transcription factors with or without CD57. Finally, in all subsets studied CD57+ T-cells were T-bet+ with or without Eomes but never Eomes+T-bet−.

6. CONCLUSIONS

The conclusions obtained from the studies presented in this doctoral thesis are presented below:

- NKT-like cells increase in frequency and polyfunctionality with the combination of age and CMV latent infection and not with CMV alone. CD57 expression in NKT-like cells is a hallmark of CMV infection and is associated with higher polyfunctionality. This results support the hypothesis of a beneficial role of CMV on the NKT-like cells responses in young age.

This conclusion was drawn from the article ***"Effect of age and latent CMV infection on CD8+CD56+ T cells (NKT-like) frequency and functionality".*** *Mech Ageing Dev.2016 Sep;158:38-45. doi:10.1016/j.mad.2015.12.003. Epub 2016 Jan.*

- CMV latent infection has a differential effect with age on the expression of CD57, CD300a, and CD161 markers on different T-cell subsets (CD4+, CD8+, NKT-Like and DN). CD57+CD300a+ T-cells expansion in young CMV-seropositive individuals suggests a relevant role of both makers in the T-cell control of CMV virus. Furthermore, there is a decrease of CD161 with age, suggesting a possible impairment of the IL-17 production in the elderly. These findings highlight the importance of taking into account both age and CMV serostatus in any clinical study regarding the analysis of T-cells and additionally, support the potential use of CD57, CD300a, and CD161 as biomarkers of immunosenescence and as possible targets for novel therapies.

This conclusion was drawn from the article ***"Differential Effect of Cytomegalovirus Infection with Age on the Expression of CD57, CD300a, and CD161 on T-Cell Subpopulations"*** *Front Immunol.2017 Jun 2;8:649. doi: 10.3389/fimmu.2017.00649. eCollection 2017.*

- CMV latent infection and age are associated with the expansion of highly differentiated CD4+, CD8+ and $CD4^{hi}CD8^{lo}$ T-cells that express differential levels of T-

bet and Eomes. This finding suggests that the expression of these transcription factors is essential for the generation and development of effector-memory and effector CD4+ and CD8+ T lymphocytes, which are involved in conferring protection against chronic CMV infection.

This conclusion was drawn from the article ***"Effect of Cytomegalovirus (CMV) and Ageing on T-Bet and Eomes Expression on T-Cell Subsets"****Int. J. Mol. Sci.****2017****, 18(7), 1391; doi:10.3390/ijms18071391.*

7. FUTURE PERSPECTIVES

Several questions remain open and the potential clinical implications of the results obtained in the present Doctoral Thesis should be further investigated.

Firstly, our results show the necessity of finding new and exclusive phenotypic markers of T-cells and for the analysis of immunosenescence in aged individuals eventually infected with CMV. This could lead to a better understanding of T-cell biology, in the context of ageing and CMV infection.

Secondly, we have still not tackled the question, as to whether CD300a+CD57– and CD300a+CD57+ T-cells display any differences regarding polyfunctionality. Nevertheless, future experiments are being developed in our laboratory that will allow us to establish whether CD300a is a polyfunctional marker of T-cells *per se*, or only if co-expressed with CD57 and how the expression of these markers is related to T-cell differentiation.

Finally, future studies are needed to explore the impact of CMV and ageing on the expression of T-bet and Eomes in other T-cell subpopulations and in relation with other new markers such as CD300a. This will allow us to understand better the role of these transcription factors in the differentiation process of T-cells involved in the response to viruses and in the development and maintenance of the immune response to different pathogens.

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9. OTHER PUBLICATIONS

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